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A CLINICAL AND EXPERIMENTAL STUDY OF THE BACTERIOLYTIC COMPLEMENT OF THE BLOOD SERUM IN VARIOLA.

RALPH L. THOMPSON.

Research scholar of The Rockefeller Institute for Medical Research.

(*From the Pathological Laboratory of the Boston Detention Hospital.*)

In studying the bacteriolytic complement content of the blood serum in variola, experimental work was done on rabbits previous and subsequent to vaccination and variolation, on apes who were successfully inoculated from cases of small-pox, and on patients during the various stages of the disease. It was at the suggestion and under the direction of Dr. W. T. Councilman that this work was done, and I wish to express to him my indebtedness and sincere thanks.

Rabbits were used for the greater part of the experimental work, as it was found that the blood serum of this animal corresponded very closely in germicidal power with human serum against the bacteria employed in the tests. Although rabbits suffer no clinical disturbance from variolation, nevertheless, certain definite reactions occur, *e.g.*, the corneal lesions, after eye inoculation, which show them susceptible to the disease. Apes were also used for experimental work, as in these animals a typical variola exanthem was obtained by Drs. Magrath and Brinckerhoff.

The clinical studies were made from patients at the Boston Detention Hospital, and represent fairly the various stages and varying types of variola.

BACTERIOLYTIC COMPLEMENT OF NORMAL, VACCINATED, AND VARIOLATED RABBITS.

Methods. — The blood for these experiments was obtained under aseptic precautions, either from the ear vein of the rabbit or from the vessels of the neck. If from the ear, the hair was clipped and the ear thickly smeared with a depilatory powder containing barium sulphide. The ear was washed subsequently with sterile salt solution to remove all traces of the powder, the vein distended by pressure from

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below, and an incision made into it with a sharp pointed pair of scissors. From this incision seven to ten cubic centimeters of blood was obtained. Control cultures were made, and, in all instances where the serum was used, were sterile. In no instance was the blood kept longer than twenty-four hours after being drawn, the animal being bled the day preceding the tests, and the blood allowed to separate in the ice-box over night. The remainder of the technic is essentially the same as that used by Longcope [1] in estimating human complement.

The first experiment was to determine the bacteriolytic power of the serum in normal rabbits and to see to what degree this varied. For this eight rabbits were used, and, as was expected, some variations occurred. It was found, except in two instances, that one cubic centimeter of unheated active serum gave complete bacteriolysis for the typhoid bacillus in six hours. Of these two, one, starting with an immediate growth of twenty-two thousand colonies, showed two hundred and eighty colonies in six hours, the other, starting with an immediate growth of nine thousand six hundred colonies, showed three colonies in the six-hour plate. Both gave sterile plates in the twenty-four-hour plants, so that bacteriolysis was complete in twenty-four hours in all cases for B. typhosus with one cubic centimeter of fresh serum.

When one cubic centimeter of heated serum was reactivated with one-twentieth cubic centimeter of fresh serum (complement) nearly similar results were obtained. The two sera that showed growth in the six-hour plates where one cubic centimeter of fresh serum was used also showed growth in the tubes where reactivation was attempted with one-twentieth cubic centimeter of the same serum. One other serum that gave sterile plates with one cubic centimeter in six hours showed six colonies in the one-twentieth cubic centimeter six-hour reactivated plate. Otherwise the results were parallel, showing that one-twentieth cubic centimeter of fresh serum was as efficient in destroying the bacteria, when supplied with sufficient amboceptors, as was one cubic centimeter of the whole serum.

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For the colon bacillus a more considerable variation occurred: one cubic centimeter of serum destroying all the bacteria in the twenty-four-hour plates in only five instances; in three instances giving a growth of two hundred and sixty, twelve hundred and eighty, and sixty colonies respectively; immediate plants being seventeen thousand, seventeen thousand, and five thousand nine hundred colonies. In reactivating for B. coli, six-tenths cubic centimeter of fresh serum was used, and this gave reactivation in only three of the five instances where sterile plates were obtained in twenty-four hours with one cubic centimeter of the fresh (unheated) serum. This seems to show that the reaction of the blood serum of the rabbit to the colon bacillus is not so uniform as it is to the typhoid organism, and that the latter is the better in general for observations of this sort. With smaller amounts of the bacteria, however, a more constant result would probably be obtained.

It may be stated that the sera that showed the most marked diminution in complement for B. coli were the same that showed the diminution for B. typhosus.

As far as could be seen from subsequent observations, the rabbits that showed this diminution of complement were in all respects normal.

The results of these observations on the normal rabbit show a marked germicidal power of the blood serum, although attended with some fluctuation, for the typhoid bacillus, which corresponds closely with the germicidal power of human blood serum for this same organism. The results with the colon bacillus are likewise similar, but apparently suffer more considerable fluctuation.

The second series of observations was conducted on vaccinated rabbits. The rabbits were vaccinated on the inner surface of the ear in two places. Tests were made as above at different intervals and no variation in the bacteriolytic complement of the blood serum that could be considered due to the vaccination was found.

The third series of observations was made on variolated rabbits. The vesicle contents, pustule contents, and disks —

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the inspissated contents of the dried lesion remaining between the layers of the skin in patients convalescing from small-pox — were used for inoculation. The vesicle and pustule vaccinations were made both on the corneas and ears, and where disks were used they were planted subcutaneously. As far as could be observed, the animals showed no clinical symptoms following these inoculations. No decrease in the bacteriolytic power of the blood serum was noted in these cases. The observations were made on four rabbits six days after variolation. With six rabbits, observations were made on the day of variolation, two days after variolation, and six days after variolation.

Observations on one rabbit were made to see if any immediate reaction occurred in the blood serum in the course of the absorption of the small-pox contagium. For the inoculation a number of disks from virulent cases of small-pox were ground in a mortar, suspended in normal salt solution, and the emulsion injected, part into the ear vein and part into the peritoneal cavity of the animal. The rabbit's blood was drawn immediately after inoculation and at intervals of thirty minutes, one hour, and three hours. The fresh defibrinated blood was used for these tests. The heated serum to which this blood was added for reactivation was from another rabbit, but previous experiment has shown us (as in the experiment of Longcope, loc. cit., with human serum) that the interactivating power of normal serum does not vary. The results in this case show that no bacteriolytic diminution of the blood occurs immediately after inoculation — sterile plates belng obtained in twenty-four hours in all cases for B. typhosus with one-twentieth cubic centimeter reactivation. There was a growth of a few colonies in the six-hour plates at the first and third bleeding.

The complement content of two rabbits was also determined two weeks after variolation, and as in the previous cases no diminution was to be noted.

During the course of this work it was not infrequent to lose rabbits from various diseases which are incident to animals in confinement. Several animals at various times

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were taken when they appeared at the point of death from certain laboratory or induced diseases, chloroformed, the blood withdrawn, and the animal subsequently killed and autopsied. The results from four rabbits so examined show a remarkable and uniform decrease in the bacteriolytic complement of the serum.

Of these rabbits, one was affected with snuffles, and autopsy showed abscesses of the right lung and liver. The second rabbit was used when at the point of death from marasmus. The third rabbit showed colon septicemia, and the fourth leptothrix infection.

All of these rabbits gave a growth of from twenty thousand to innumerable colonies in the twenty-four-hour plates, and a correspondingly large growth in the six-hour plates for B. typhosus with one cubic centimeter of fresh serum and with one-twentieth cubic centimeter reactivation.

These latter observations are of interest as coinciding with the decrease in bacteriolytic complement found in individuals in the course of disease.

To summarize briefly, we may say that the bacteriolytic complement of normal rabbit blood serum is closely allied to the bacteriolytic complement of normal human blood serum for the typhoid and colon bacillus.

Normal rabbits show some individual variation in complement for the typhoid bacillus, and more marked fluctuation for the colon bacillus.

Vaccination and variolation do not affect the bacteriolytic complement of rabbit's serum for the typhoid and colon bacillus.

The blood serum of rabbits in the last stages of certain fatal diseases shows a marked diminution of bacteriolytic complement.

THE BACTERIOLYTIC COMPLEMENT IN VARIOLATED APES.

For the study of the bacteriolytic complement of the ape I was enabled, through the kindness of Dr Magrath and Dr. Brinckerhoff, to observe the blood serum of two species of

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ape (rhœsus and macacus) during variolation. The influence of the complement on typhoid bacilli was studied under the same conditions as in the human and as in rabbits.

Observations on the bactericidal power of the blood of the ape are of interest, since the blood serum of this animal so nearly approaches the human in various respects, *e.g.*, its precipitin reaction, and since a true variola may be obtained in this animal by inoculation.

Experiments were first made with normal animals, and it was found that, as in the human, one-twentieth cubic centimeter of fresh (unheated) serum was sufficient to reactivate one cubic centimeter of heated serum so as to give sterile plates with the typhoid bacillus in from six to twenty-four hours. With this as a standard, the following observations were made. (The animals are catalogued in the same order as the experiments appear in the paper by Drs. Magrath and Brinckerhoff, and I am indebted to them for permission to quote from their observations as to the clinical conditions, temperature, blood counts, etc., which they made.)

Ape II. — Macacus. Showed vesicle formation five days after inoculation. Well-marked "take" four days later. Fever from fifth to fourteenth day. Marked leucocytosis from tenth to thirteenth day. Reinoculation after healing of lesion without a "take." Later vaccination attempted without result.

Observations were not made on this animal during the progress of the first inoculation. Beginning a week before the second inoculation, observations were made every few days until after the second inoculation and the vaccination (twenty-three days). Sterile plates were obtained in all instances from twenty-four-hour tubes. In one instance there was a growth of six colonies in one of the six-hour tubes with one-twentieth cubic centimeter reactivation. This was the day following inoculation.

Ape III. — Macacus. Double inoculation; the second four days after the first. Marked constitutional reaction, anorexia. Leucocytosis forty-eight hours after each inoculation. First inoculation showed crust formation; second inoculation elevation and redness and slight vesicle formation. Parasites not demonstrated histologically.

Five observations of the complement were made on this ape. The first the day before the inoculation; the second forty-eight hours after inoculation, when there was a leucocytosis of 30,000.

The first of these observations shows a growth of eight hundred and orty colonies in the twenty-four-hour plate. The second gave innumerable colonies in the twenty-four-hour plate. The subsequent examinations

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also showed a diminished complement, although on the day of autopsy a sterile twenty-four-hour plate was obtained.

The animal was in poor physical condition at the start, and showed more marked constitutional symptoms after inoculation than did any of the other animals. He refused food and appeared weak and sick. Autopsy, however, did not show any specific disease.

APE IV. — Macacus. Production of general exanthem eight days after inoculation. No constitutional reaction. Leucocytosis seventh and eighth days. Cytoplasmic forms of the parasite found histologically. Four observations were made on the complement, the first previous to inoculation, the second the fourth day, when there was a leucocytosis of 15,000, and the others with the appearance of and subsequent to the eruption. There was no diminution of complement observed at any time. Sterile plates being obtained in eighteen hours in all cases with one-twentieth cubic centimeter reactivation.

APE V. — Rhœsus. Inoculated with pustular contents into jugular vein. Died of streptococcus septicemia forty-five hours after inoculation.

Observation before inoculation showed normal complement content.

The second observation made twenty-four hours after inoculation, and the third made just before death, showed innumerable colonies in the twenty-four-hour plates, both B. typhosus and streptococci being present.

APE VI. — Rhœsus. Well-developed lesion six days after inoculation. No constitutional reaction (animal natural and eating well). One degree elevation of temperature. Leucocytosis of 30,000 before inoculation; 15,000 on fifth and sixth days. Parasite present in primary pustule. Observations continued throughout the experiment showed always a normal complement. Sterile plates being obtained in eighteen hours with one-twentieth cubic centimeter reactivation in all cases.

To sum up briefly, we have five apes, in four of which variola inoculata was obtained. Of these four, only one showed marked constitutional reaction, that is, decided weakness, depression, and anorexia, although there was an average elevation of temperature of two degrees Centigrade in the inoculated animals. There was a leucocytosis in all cases, which did not apparently affect the complement content. There was one case of streptococcus septicemia.

Observations on the blood serum show that the production of experimental variola causes no diminution of the bacteriolytic complement content in this animal.

The diminution of complement in apes III. and V. tend to confirm the experiments previously made, which show that lowered vitality and terminal infection cause a diminution in the germicidal power of the blood.

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Ape.	Reactivation.	Immediate.	Six hours.	Twenty-four hours.
II. Variola inoculata.	1/20 cc.	16,000 to 32,000.	6 to sterile.	Sterile (all instances).
III. Variola inoculata, anorexia, weakness.	1/20 cc.	14,000 to 30,000.	3,000 to 200.	840 to innumerable. (One observation sterile with 1/10 cc. reactivation.)
IV. Variola inoculata.	1/20 cc.	6,000 to 12,000.	Sterile.	Sterile.
V. Streptococcus septicemia.	1/20 cc.	9,000 to 16,000.	14,000 to 20,000.	Innumerable.
VI. Variola inoculata.	1/20 cc.	12,000 to 16,000.	Sterile.	Sterile.

THE BACTERIOLYTIC COMPLEMENT IN VARIOLA.

In undertaking the study of the complement content in human variola, one of the first difficulties that presented itself was to obtain sufficient amounts of blood from each patient to carry out the number of observations that it seemed necessary to make in the different stages of the disease. In an infectious disease of this sort, where secondary infections are so common, it seemed hardly justifiable to open several times, or even once, one of the larger veins, and, moreover, the extensive edema and distribution of the skin lesions in many of the cases would render the opening of an arm vein a rather dangerous and delicate surgical operation. While there are methods for determining the complement content of the blood with small amounts of serum, notably those of A. E. Wright[2] and Ainsley Walker,[3] they seemed to us less satisfactory than the technic described by Longcope (loc. cit.) in his study of chronic disease. This latter, however, which seemed to be the most satisfactory in many ways, presented the difficulty of requiring considerable amounts of blood serum in the carrying out of its details.

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A consideration of this method shows that the greater part of this serum is used after inactivation to supply sufficient amboceptor for the complement tests, and that in reality very minute amounts of the fresh patient's serum is necessary for the destruction of the B. typhosus, provided one has sufficient amboceptor. If, therefore, any means could be devised whereby an amboceptor, other than that of the patient himself, could be substituted that would link with the bacteria on the one hand, and with the complementary body on the other, a method would be obtained whereby extended observations could be carried on in the course of a given disease, because of the fact that only a very minute amount of the patient's serum would be required for the test.

Longcope (loc. cit.) showed that heated serum of different individuals could be substituted indifferently in reactivating a given complement, so that it made no difference whether a given complement was added to the heated blood serum of that same individual or to the heated blood serum of another individual in obtaining bacteriolysis. If, then, different human amboceptors would link with a given complement so as to give bacteriolysis, the thought suggested itself: Why might not certain animal sera contain an amboceptor that would fit the haptophore of a human complement, and accomplish the same result?

The previously described observations on the complement content of the rabbit showed a remarkable parallel between the reactivating power of rabbit and human sera. The experiments of Grünbaum and others on the precipitins show a close analogy between the blood of man and of monkey.

Therefore a series of experiments were undertaken to determine whether or not sera of other animals when deprived of complement could be substituted for heated human serum in supplying an amboceptor for human complement. The result of these preliminary experiments show conclusively that the amboceptors of at least three different blood sera, namely, those of the monkey, of the rabbit, and of the horse, will combine with the complement of human serum, giving

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bacteriolysis for the typhoid bacillus in identically the same manner as when the heated and unheated serum of the same person is used for the test. Therefore complement estimates can be made as often as desired without danger or disturbance to the patient, as a simple puncture of the ear yields enough serum for this test.

An understanding of this phenomenon may, perhaps, be aided by enlarging upon the simile of Fischer, who likens the cell or bacterium to a lock, the amboceptor to a key, and the complement to the hand that turns the key. Bordet showed that the heated serum (amboceptor) could be reactivated so as to produce hemolysis by the addition of complement from various sources. In other words, given the lock and key, almost any hand could turn the key. On the other hand, the fact that several different keys might fit the same lock had not been given consideration. But that such is the case our experiments show. The only thing being necessary is to have keys of very similar construction, and in the case of human and some of the animal sera, the similarity is so near that the lock may be turned by either of these keys, provided there is strength enough in the hand (complement) to manipulate the key. To complete this analogy, bringing in the importance of a deficiency of complement as a cause of disease, we may say that the hand is not strong enough to turn the key, and thereby the door remains unlocked. Such may be the case in certain individuals; there is a deficiency of complement, and, though we have the lock and key, immunity fails to close the door, and the individual suffers the results of the invasion of a pathogenic organism.

In following this analogy, however, it is well to remember that the importance of only one of the factors concerned in bacteriolysis is emphasized, and that in infectious processes we have many things other than the complement of the blood to consider. The complement content is, however, of marked importance, and a deficiency of the same, we may say in the light of our present knowledge, cannot exist without some danger to the individual.

Technic. — In the beginning of these experiments rabbit serum was used for reactivation, as it could be easily obtained, the blood of one large rabbit being sufficient for a considerable number of observations. Later on horse serum was substituted for the rabbit's serum, as it was found to answer the purpose, and saved the time necessary for obtaining rabbit's blood. Normal horse serum was kindly furnished me through the courtesy of Dr. Kinyoun.

This facilitated the work considerably. A point in regard

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to the horse serum is that it was found necessary to heat it for one and one-half hours at 57°–58° C. to completely destroy the complement.

Vedder [4] has noted that when a small amount of complement is added to normal salt solution (one-twentieth cubic centimeter of complement to one cubic centimeter NaCl) that some bacteriolysis takes place. That is to say, the salt solution plays to some extent the same part, apparently, as does the immune body in heated serum when complement is added to it, or the small amount of immune body present (for complement is not to be obtained pure) acts in some way in the presence of salt solution. The same phenomenon has been noted in my experiments, but in no instance has there been sufficient bacteriolysis where complement was added to salt solution to compare with the results where heated serum was reactivated. The term "complement" is, of course, at present only relative, as no satisfactory way to separate it wholly from the bacteriolytic amboceptors is at hand. The appearance of such a method will doubtless clear up many problems connected with work of this sort.

It might be stated that, in inactivating a large number of tubes of serum at the same time, they should not be closely packed, but should be so separated as to assure the full force of the heat reaching each tube immediately on being placed in the oven; otherwise tubes may be found which will give sterile plates without reactivation.

The steps of the process of the complement determination were as follows:

1. Obtaining rabbit's serum. — The animal is chloroformed and the carotid exposed under aseptic precautions. The artery is then ligatured above, a sterile capillary tube connected with a sterile flask inserted into the artery, the clip removed, and the blood allowed to flow into the flask. The blood is placed in a cool place until the serum has separated. When the separation is complete, the serum is transferred by means of a sterile pipette into small tubes, one cubic centimeter to each tube. The tubes are then placed in an incubator at 57° C. for one hour to destroy the complement. The serum is now ready for use, or it may be kept for weeks, if necessary, in a cool place.

2. Obtaining patient's blood and reactivating rabbit's serum. — The blood for the complement observation is obtained from the patient's ear in

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the same manner as is generally used in procuring blood for a Widal test. Sufficient blood may be obtained by a single puncture, as one-fourth cubic centimeter is ample amount for a test. Tubes for this may be readily made from glass tubing. They should be sterile and plugged with cotton. The serum separates quickly, especially if a sterile platinum wire be run between the clot and the glass. When the serum has separated, the necessary amount (one-twentieth cubic centimeter) is transferred by means of a sterile capillary pipette into the tube containing one cubic centimeter of inactivated rabbit's serum. The serum thus obtained was sometimes used immediately on its separation from the clot (about one hour after withdrawal), or was withdrawn at night and used the next morning (twelve to eighteen hours after withdrawal). Walker (loc. cit.) has stated that a slight fluctuation in the complement is incident to the time the serum is used after withdrawal. These slight fluctuations, which he gets in the complement content from hour to hour, are very different from the marked changes observed in the course of investigations of disease, and would not be notable in an investigation of this sort, where changes when they occur are much more marked.

Controls were made between the serum used immediately after withdrawal and the serum which had stood for twelve to eighteen hours, and no differences that would affect the general results here given were observed.

3. Preparation of typhoid emulsion. — The typhoid emulsion was prepared by adding to a tube containing three cubic centimeters of sterile (.85 per cent) salt solution, a small amount of the growth from a twenty-four-hour streak culture of B. typhosus on agar or Lœffler's blood serum. The amount used is necessarily not always the same, but such an amount that one loopful of the mixture (salt solution + B. typhosus) will give a plate of from ten thousand to thirty thousand colonies can be uniformly obtained. A double loop was used in transferring this mixture to the reactivated rabbit serum, three double loopfuls (approximately one-fiftieth cubic centimeter) being added to the serum.

4. Plating. — Having now the preparation of amboceptor (heated rabbit serum) plus complement (patient's serum) plus B. typhosus emulsion, the mixture should be thoroughly shaken and two double loopfuls (or four single loopfuls) added to the agar and planted. The agar should be very thoroughly shaken before plating to insure a uniform growth of colonies. The tubes and plates are then placed in the incubator, 37.5° C., and new plates are made at the desired intervals.

5. Counting colonies. — The exact estimation of the colonies in the plate where there is a considerable growth is not of particular importance, as when the colonies run into the thousands a few hundred one way or another make no particular difference, but when there is a scant growth, showing that more or less bacteriolysis has taken place, they should be counted carefully. The colonies were estimated in the cases where there was considerable growth by counting several square centimeters at different parts of the plate, and from them making an average of the number of

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colonies on the whole surface. Where there was a scant growth the entire surface of the plate was counted. A No. 3 ocular was used to facilitate the counting process.

Having in mind the idea previously outlined, the first observation was to see if bacteriolysis could be obtained for B. typhosus by reactivating one cubic centimeter of heated rabbit serum with one-twentieth to one-tenth cubic centimeter of fresh serum from a normal individual. Such was found to be the case, bacteriolysis being complete in six hours with both these reactivations on two healthy individuals from whom Widal amounts of blood were obtained for the test. While this was not conclusive evidence that the rabbit's serum furnished an amboceptor parallel to the human serum, nevertheless it pointed to such being the fact, and so the following test was performed:

R. T. Healthy male, bled from arm (twenty-five cubic centimeters). Serum separated from clot twelve hours after withdrawal and pipetted into tubes, one cubic centimeter in each tube. These tubes placed in the incubator at 57° C. for one hour. Tubes containing one cubic centimeter of freshly drawn rabbit's serum (A) and rabbit's serum (B) two weeks old that had been kept in a cool place were inactivated at the same time. These tubes were then reactivated with one-twentieth to one-tenth cubic centimeter fresh, unheated (R. T.) serum, and an equal quantity of suspension of B. typhosus added to each. The control plant of one loopful of this suspension gave a growth of thirty thousand colonies.

The results of this experiment show an accurate parallel between the tubes, whether heated human or rabbit serum was used for the intermediary body, as may be seen from the following:

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(R. T-)	Immediate.	6 hours.	9 hours.	24 hours.
1 cc. heated human serum plus 1/20 fresh human plus B. typhi,	12,000	Sterile	Sterile	Sterile
1 cc. heated human serum plus 1/10 fresh human plus B. typhi,	9,600	"	"	"
1 cc. heated rabbit serum A plus 1/20 fresh human plus B. typhi,	13,200	"	"	"
1 cc. heated rabbit serum A plus 1/10 fresh human plus B. typhi,	8,400	"	"	"
1 cc. heated rabbit serum B plus 1/20 fresh human plus B. typhi,	6,000	"	"	"
1 cc. heated rabbit serum B plus 1/10 fresh human plus B. typhi,	12,000	"	"	"
1 cc. unheated human serum plus B. typhi	15,000	"	.....	"
1 cc. unheated rabbit serum plus B. typhi	15,600	"	.....	"
1 cc. heated human serum plus B. typhi	12,000	30,000	.....	Innumerable
1 cc. heated rabbit serum plus B. typhi	14,000	32,000	.....	Innumerable

The test was carried out in the same manner, substituting heated monkey serum (rhœsus) and heated horse serum in place of the heated rabbit serum. The results were the same as in the first experiment. Either of these latter sera serving to replace the heated serum of the individual tested, and giving sterile plates in six and twenty-four hours when reactivated with one-twentieth cubic centimeter of the normal human complement containing serum. That this destruction of the bacteria would not take place in the heated animal serum alone was shown by the fact that when plants were made from these tubes of heated serum before reactivating with fresh human serum there was always a growth of from thirty thousand to infinite colonies in the twenty-four-hour plates.

Having shown, then, that these heated sera could be used in quantitating the complement of an individual, a series of

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observations were made on apparently healthy individuals to see what variations might exist. Heated rabbit serum was used for the intermediary body. It has already been shown by Wright (loc. cit.) and by Longcope (loc. cit.) that there is a considerable variation in the complement content in normal people, depending, in their cases, especially upon overwork and anxiety These observations bear out these facts, and show that in individuals who may in no way be called diseased a considerable diminution in the complement content may occur, which can at times be accounted for, but sometimes not.

These observations were made on twelve healthy individuals about the hospital, including physicians, nurses, and laborers. Of these, seven showed a high complement content at each observation, several observations being made on each during a period extending over six weeks, all giving sterile plates in six hours and twenty-four hours, with one-twentieth cubic centimeter of serum.

In two other cases the observations were of interest. In the first of these, both one-twentieth cubic centimeter and one-tenth cubic centimeter inactivation failed to give sterile plates, even in the twenty-four-hour plants. The man on whom the observations were made had been doing exacting work for some time, with loss of sleep and loss of air and exercise. Two days after the observation was made, two small streptococcus infections appeared on this man's hand. With a relief from work and the healing of the infections, it was found that one-twentieth cubic centimeter of his serum gave sterile plates in six hours. The other of these cases had shown high complement on several observations, one-twentieth cubic centimeter giving sterile plates in six hours, but during the course of an attack of influenza the complement was found diminished, one-twentieth cubic centimeter of his serum failing to give sterile plates in twenty-four hours.

Of the three remaining cases of this group, one observation was made in two cases: the first, a girl, aged five years, who failed to give sterile plates in twenty-four hours, when either one-twentieth cubic centimeter or one-tenth cubic centimeter

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of her serum was used for reactivation. Though in good health at the time, she had had an attack of broncho-pneumonia several weeks previously. The second of these cases, a male, middle-aged, failed to give sterile plates with one-twentieth cubic centimeter or one-tenth cubic centimeter of his serum. The last of this group was a young, healthy Irish girl, on whom a number of observations were made, but in no case was such reactivation obtained, with one-twentieth or one-tenth cubic centimeter of her serum, as to give sterile plates in twenty-four hours. In this latter case, especially, no apparent reason presents itself for the diminution of the complement, and we must conclude, therefore, that in certain individuals this complement diminution exists. It makes it necessary, then, in studying any given disease, to take the possibility of such conditions into account before drawing too definite conclusions as to the disease being always the cause of the diminution of complement.

Assuming, then, from the above observations, that in the greater number of normal individuals one-twentieth cubic centimeter of their fresh serum would reactivate one cubic centimeter of heated rabbit serum so as to give sterile plates in from six to twenty-four hours, a series of observations were undertaken in patients afflicted with variola to see what changes, if any, might occur in the course of this disease.

It is to be understood that in studying a disease like variola, unless the epidemic be unusually severe, one cannot pick material, but must use whatever he is fortunate to find at his disposal at the time. In this investigation a fairly representative group of cases of the different forms and different degrees of severity of the disease came under observation. In all, fifteen cases were studied and may be grouped as follows:

1. Variola vera, abortive type. Majority of lesions not coming to pustulation, six.

2. Variola vera, mild, but disease running complete course, five.

3. Variola confluens, all with more or less secondary infection, three.

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4. Purpura variolosa, one.

A complete account of these cases has previously been published [5] and here only a summary of the results will be given.

In all instances where an observation of the patient's blood was made early in the course of the disease a low complement content was found. The nearest approach to an exception to this rule is in one case where the first observation made on the fifth day of the disease showed a normal complement.

In the abortive cases and the cases running through the entire course of the disease, but unattended with secondary infection, there is a rapid return of the complement to the normal standard. This rise of complement content in the abortive cases is in coincidence with the beginning involution of the lesions. In the cases running through pustulation and unattended with secondary infection, this rise may be noted in the later pustular stage or during the early stage of dessication. One may, perhaps, say in some cases that an absence of secondary infection may be predicted when one gets a return of the complement to normal in the early pustular stage; but a prediction that secondary infection would occur could not be made at this time from a low complement, for in some of the mild cases complement may be low throughout the entire course of the disease, just as it may be low in certain normal individuals.

Two cases offer an interesting comparison as regards the complement content reduction being in inverse ratio to the severity of the disease. Two patients, a boy and a girl, both infected from the same case, entered the hospital at the same time. At the time of their entrance, and until the vesicular stage was well developed, there was practically no difference to be observed in the severity of the two cases. The girl, however, did not go on to the pustular stage. Her lesions aborted and she quickly returned to health. Her complement was normal throughout.

The boy, on the other hand, continued on through a well-marked pustular stage, though without secondary infection,

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his case being considerably more severe and more prolonged than that of the girl. His complement showed a reduction until the late pustular stage, when just before involution of the pustules it rose to normal and so remained.

In the cases attended with secondary infection (from which streptococci were isolated) there was always a low complement content — the diminution continuing throughout convalescence in all of the observed cases, and the patient being discharged while the complement content was still low. In one case there was some increase in complement content at the last observation, though not a complete return to our arbitrary normal standard.

That there should be rapid rise of complement in the uncomplicated cases of variola seems natural from the resistance that the individual offers to this disease. In variola the recuperation of the patient is remarkable. With the passing of the lesion the patient in most cases is well in all senses of the word, and that a normal complement should accompany this quick return to health is not remarkable.

That a low complement content should persist when secondary infection occurs is of considerable interest, in that it bears out the work previously done in showing that a low complement is coincident with secondary infection (Longcope, loc. cit.). In this connection the case of hemorrhagic smallpox is of value in establishing the conclusion that a low complement is coincident with terminal infection. This case suffered a progressive complement diminution from the time of her entrance to the hospital until her death.

Since the completion of this work, Perkins and Pay[6] have published observations of the bacteriolytic complement in variola. Their observations, for the most part, were made on fatal cases; the serum being taken after death. In all their severe cases attended with secondary (streptococcus) infection a diminution of complement was also noted.

To briefly summarize these observations, then, we find in variola:

1. A diminution of complement content in the early stage of the disease.

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2. A rapid return of the complement content to normal in the cases unattended with secondary infection. This return is seen in the abortive cases with the beginning of the involution of the lesions; in the others, during the late pustular or early dessicating stage.

3. A failure of the complement content in its return to the normal in cases in which there is secondary infection.

4. A progressive continued diminution in the complement content when the case is complicated by terminal septicemia.

An interpretation of these results may be aided by a brief consideration of the disease, variola. In variola we have a clinical entity which involved two processes. (1.) Infection by a definite specific contagion. (2.) A secondary local or general pyogenic process, which may or may not be present, but which, if present, may offer the gravest consequences to the patient.

From our observations we must conclude: That infection with the specific contagion of variola causes a diminution of the bacteriolytic complement of the individual, and that this diminution is more or less dependent upon the severity of the attack. Also that a mild infection, in which there is very little clinical disturbance, might occur without such complement diminution being noticed. This should be inferred from the fact that in one of our mild cases no diminution was present at the time the patient entered the hospital.

Given an individual attacked by a definite specific contagion, it is reasonable to suppose that this individual will exert all possible resistance against the contagion. Provided this resistance is of avail, it is shown by a rapid return of the complement to the normal standard. Provided, however, the resistance of the individual is for some reason weak or the infection is unusually severe, this rise of complement to normal does not take place, and we have a patient entering the pustular stage of variola with a diminution of the bacteriolytic power of the blood serum.

Given, then, a lowered complement and a supply of

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pyogenic organisms (streptococci were isolated from the lesions of practically all of our cases), it is easy to see how variola may be complicated by a local or general pyogenic process; and from the fact that the complications due to pyogenic infections, and that death from streptococcus septicemia were, in the cases observed, always accompanied by a continued lowered complement content, we must conclude that this lowered complement content in variola is the cause of such infection taking place and is of danger to the individual.

The foregoing observations emphasize the fact that systematic studies of the bacteriolytic power of the blood serum carried out in various diseases would be of value. Especially does this seem to be true from a prognostic standpoint. The rapid advances that are now being made both in the technic and the understanding of the phenomenon of bacteriolysis should make such observations of practical importance.

SUMMARY.

1. Most normal individuals have a high bacteriolytic complement content. This complement content is, however, remarkably fluctuant, conditions of lowered vitality, due either to fatigue or infection, causing a diminution which may under proper conditions be promptly restored.

2. Certain individuals show on repeated examination a low complement content, the cause of which may not be evident.

3. The blood serum of rabbits and of apes shows under normal conditions a high bacteriolytic complement content similar to that in the human. The complement in these animals is not diminished by vaccination or variolation, but is diminished in certain fatal diseases, notably septicemia.

4. In individuals suffering from variola there is a diminution of complement in the early stage of the disease, with a return to normal in the cases in which there is no secondary infection. In cases in which there is secondary infection and terminal septicemia there is a continued diminution of complement.

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A STUDY

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NECROSES OCCURRING IN THE LIVERS OF EXPERIMENTAL ANIMALS AFTER INOCULATION WITH HOG-CHOLERA BACILLI

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(Austin teaching fellow in Comparative Pathology, Harvard Medical School)

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A STUDY OF THE NECROSES OCCURRING IN THE LIVERS OF EXPERIMENTAL ANIMALS AFTER INOCULATION WITH HOG-CHOLERA BACILLI.[1]

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In the livers of rabbits and mice that have died from the effects of inoculations of hog-cholera bacilli there occur small characteristic grayish or yellowish white patches involving one or more lobules. These patches are areas of coagulation necrosis, and while they vary considerably in number and size, they are of extremely constant occurrence in such animals. They attain, usually, their greatest size in the thin dependent borders of the liver. The superficial lobules are most often affected, yet at times the deeper ones are also involved.

These necroses were first described by Klein, who observed them in the livers of rabbits and mice inoculated either with tissue or cultures taken from hogs that had died of pneumo-enteritis or pig typhoid. He described both a bacillus and a micrococcus in the necrotic areas, but he was inclined to consider the bacillus the more important organism of the two.

Somewhat later Theobald Smith showed that these peculiar liver necroses constantly followed the inoculation of rabbits and mice with a fatal dose of his hog-cholera bacillus, provided the animals survived sufficiently long (seven or eight days).

Both Klein and Smith considered these patches as areas of coagulation necrosis due to a plugging of the vessels; but neither at the time was able to show how this plugging occurred, nor did they give any detailed account of the necroses.

About four years ago Dr. Smith, in looking through some

[1] Received for publication Feb. 3, 1903.

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sections of mouse liver, noticed in some of the vessels adjacent to the necrotic areas, thrombi of a peculiar nature. They were apparently balls of more or less fused and altered red blood corpuscles.

This led him to suggest to me that I make a study of the necrotic changes occurring in the livers of animals inoculated with hog-cholera bacilli.

MATERIAL AND TECHNIQUE. — In order to trace the origin and development of the lesions, a series of mice was inoculated and certain of them killed at intervals. Mice were selected for these experiments because of their marked susceptibility to the disease and the constancy with which the necroses appear in their livers. In this series only tame mice were used, but in the remainder of the work both wild and tame ones were used.

The culture employed was one of a bacillus isolated from an outbreak of hog-cholera by Dr. R. R. Dinwiddie in Arkansas. It was of the α type of Smith, which type he describes as the most virulent. Although it had been in the laboratory about two years, and had been passed through a series of guinea pigs, it was very virulent to rabbits, and quite so to mice.

The mice were inoculated with .02 cc. of a twenty-four-hour bouillon culture, so diluted with sterile salt solution that 0.1 cc. was injected. This amount had previously been determined as about as small a dose as could safely be relied upon to produce death.

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TABLE I.

	Sex.	Weight.	Killed hrs. after injection.	Red thrombi.
Mouse 23	Male	18 grms.	8 hours	—
" 16	"	14 "	21 "	—
" 18	"	10 "	21 "	—
" 22	"	16 "	20 "	—
" 20	"	15 "	46 "	—
" 21	"	12 "	46 "	—
" 12	"	19 "	72 "	—
" 13	"	14 "	72 "	—
" 10	"	24 "	95 "	—
" 11	"	18 "	95 "	+
" 17	Female	15 "	119 "	+
" 19	"	17 "	119 "	—
" 14	"	23 "	143 "	—
" 15	"	24 "	143 "	—

Unfortunately the mice used in this series varied considerably in age and weight, and on this account, in resistance, hence the results were not so uniform as desirable. Nevertheless they served the purpose fairly well.

In addition, the lesions were studied in twelve other mice that had died from the effects of injections of varying amounts of the same, and three other cultures isolated from different epizoötics in swine and furnished by Dr. Smith.

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TABLE II.

	Sex.	Weight.	Culture.	Dose	Died.
Mouse 1....	Wild male	Adult	Arkansas ∝	0.05 cc.	5½ days
" 2....	" female	"	" "	"	7½ "
" 3....	Tame male	16 grms.	" "	0.025 cc.	7½ "
" 4....	" "	16 "	" "	"	5½ "
" 5....	" "	20 "	" "	"	5½ "
" 6....	" "	17 "	" "	0.0125 cc.	12[2] "
" 7....	" "	22 "	" "	"	7½ "
" 8....	" "	20 "	" "	0.025 cc.	7½ "
" 26....	Wild female	Adult	Massachusetts ∝	0.05 cc.	6½ "
" 27....	" "	"	Maryland ∝	"	7 "
" 31....	Wild	"	Non-motile [1]	0.30 cc.	6½ "
" 32....	"	"	"	0.12 cc.	8½ "
" 33....	"	"	"	0.06 cc.	5½ "

[1] Centralblatt f. Bakt., 1889, Bd. xxv, s. 241.
[2] Chloroformed.

Several other uninoculated mice, both wild and tame, were examined as controls.

From some experiments going on in this laboratory with this same disease I was able to obtain considerable rabbit and guinea-pig material. As visible liver necroses occur but rarely in guinea-pigs, little attention was given them, but so far as I have examined such as did appear, they resembled in general those found in mice and rabbits. The rabbit material was composed wholly of tissue from rabbits that had succumbed to the disease. The time they survived after inoculation varied from five and one-half to eleven and one-half days. While the necrotic processes taking place in rabbits are essentially the same as those in mice, they are not so easily followed, and for this reason the description following will apply for the greater part to mice, the chief points of difference being noted.

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Tissue from chloroformed animals was fixed at once. But as those that were allowed to die of the disease almost invariably died during the night, the tissue from them was not so fresh, yet no appreciable difference could be seen.

The fixers tried were Zenker's, Flemming's, formalin, absolute alcohol, and acetic-sublimate. For general purposes Zenker's was the most useful.

Eosin followed by Unna's polychrom-methylene blue gave the most satisfactory stain. Delafield's hematoxylin with Van Gieson's picro-acid fuchsin, Ehrlich's triacid, safranin, and Weigert's fibrin stain with alum carmine were also used with good results.

The sections studied varied from two to six microns in thickness.

PHAGOCYTIC-CELL THROMBI. — On examining sections of the liver of a mouse killed twenty-four hours after an injection of hog-cholera bacilli, one notices clumps of large cells lying in the distended capillaries. These clumps are, perhaps, more numerous just beneath the capsule of the liver and the walls of the veins. The interlobular connective tissue, also, in certain regions is brought into prominence owing to the large number of these cells in its interstices.

The size of the cell foci varies much. They may consist of but three or four cells or of a considerable number. As a rule, however, they are, at this stage of the disease, yet of small size. The cells composing them may stain deeply with methylene blue in one clump, while in another neighboring one they may take a feeble eosin stain.

These cells are large mononuclear cells with a relatively large, generally eccentrically placed vesicular nucleus. The shape of the nucleus in the younger forms is oval or reniform. As the cell grows older, however, the nucleus becomes more irregular, assuming often a horseshoe or ring form (Fig. 1). The nucleus consists of a distinct, deeply-staining membrane with enclosed feebly-staining karyoplasm in which lie chromatin masses of varying size and shape. The cytoplasm of the newly formed cells stains deeply with most basic dyes,

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especially so with methylene blue. With polychrom-methylene blue it is metachromatic, assuming a violet tint. The cytoplasm in structure is finely reticular, resembling that of the large lymphocytes. Occasionally a few fine granules may be made out by certain staining methods.

These cells are of low vitality, and very soon begin to show degenerative changes. In cells with the horseshoe or ring-shaped nucleus, the enclosed portion of cytoplasm first shows this change. It takes a feeble eosin stain, while the remainder of the cytoplasm still stains with methylene blue. Later the whole of the cytoplasm is affected, and stains no longer with the basic dye, but takes a feeble eosin stain. (Fig. 2.) The degenerative changes in the nucleus are shown in its greater irregularity and the lessened intensity with which it takes the dye.

The form of cell usually met is round or elliptical, yet ameboid forms are not uncommon. That they are considerably larger than other blood cells, the following measurements from a blood smear fixed by heat will show:

Large mononuclear cells, 13.5 μ — 18.7 μ average diameter.

Polymorphonuclear leucocytes, 11.23 μ.

Small lymphocytes, 7.8 μ.

Red blood corpuscles, 6.59 μ.[1]

These large mononuclear cells possess phagocytic powers, and occasionally one finds within them red blood corpuscles, lymphocytes, and polymorphonuclear leucocytes. Bacteria have been observed in these phagocytes of the rabbits, but not in those of the mouse. The phagocytes of the rabbit are identical in structure with those of the mouse, except that in a considerable number of cells one sees many fine acidophile granules about the size of the finer eosinophile granules of the leucocytes.

One may now readily identify these cells with those phagocytic cells observed by Freidreich, Wagner, Billroth, Mallory, and others in typhoid. Flexner notes their presence in rabbits poisoned by abrin and ricin. Mallory pictures similar cells in the guinea pig. And more recently, under

[1] Average diameter, according to Gulliver, being 6.7 μ.

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the title of "Endothelial Phagocytes of the Tonsillar Ring," Goodale describes cells of the same sort. The question immediately presents itself, Where do these cells originate?

In many mice the peritoneal endothelium of the surface of the liver shows marked signs of proliferation. Swelling of the nuclei and mitotic figures is common. Then again, the frequent presence of cell clumps near the surface of the liver argue in favor of the origin of a considerable number from this source. It is probable, also, that they arise from the endothelial cells of the lymph and blood-vessels of the interlobular connective tissue. In the rabbit they are plainly seen arising from the endothelial lining of both the hepatic and portal veins. In the mouse, however, this mode of formation is not so frequent, and it is safe to conclude that by far the greater number arise in the spleen and are carried into the capillaries of the liver. For on examining the spleen of an infected mouse, large masses of these cells in active division may be seen adhering to the muscular trabeculæ. These places of origin correspond with those given by Mallory for the phagocytes of typhoid. Recently Beattie has shown that cells of this sort arise from the endothelium of the omentum in guinea pigs. These cells are not confined to the portal system, but are found also in the general circulation, in the heart, the vessels of the kidney, adrenal, and skin, and cells that one cannot distinguish from them may be found in smears from a drop of blood from the tail. In fact, it is impossible to differentiate them from the large mononuclear leucocytes, with which, I believe, they are identical. Associated with these cells in the masses blocking the capillaries, we find desquamated endothelial cells, plasma cells, lymphocytes, and polymorphonuclear leucocytes, and less often, but not at all infrequently, eosinophile cells. These cell thrombi tend to increase in size by the addition of cells carried to them by the blood stream, and, to some extent, by multiplication of the cells composing them.

Having traced the history of the phagocytic cells we may now recognize as youngest those cell clumps in which the cells composing them stain with basic dyes (Fig. 1). With

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age the cells lose their power to stain with methylene blue and take a feeble eosin stain (Fig. 2). Farther signs of degeneration rapidly become apparent, the cells losing their outlines, their nuclei shrinking and fragmenting. Many show fatty degeneration.

Within forty-eight hours fibrin is deposited on and among the cells, binding them closely together. This fibrin deposition may take place throughout the cell clumps or be confined to but one side. As time passes, more fibrin is deposited, the cells disappear by liquefaction, and by the seventh day only a network of fibrin containing cell fragments remains (Fig. 3).

The formation of cell thrombi is not confined to the first few days, but takes place continuously up to the time of death. In animals that have been allowed to die, these clumps are found in all stages of formation and degeneration. In those mice that showed signs of recovery, invasion of these areas by polymorphonuclear leucocytes took place, but as none were allowed to fully recover, the ultimate fate of the lesions could not be determined.

The liver cells, as one would suppose, are not unaffected by this plugging of the capillaries by phagocytic cells, and they show marked necrotic changes. At the time of the first appearance of cell clumps, beyond a general swelling of the liver cells producing a narrowing of the capillaries, no other lesion is seen. Yet even before the phagocytes begin to show signs of degeneration, the adjacent liver cells begin to go to pieces. The nucleus fragments (karyorhexis), the cell wall nearest the thrombus, ruptures and the cell contents escape. Occasionally a somewhat different change occurs, — it is the more common in rabbits. The cytoplasm of the liver cells which normally in both animals stains a neutral tint, and contains basic staining particles on the side nearest the thrombus, shrinks, becomes hyalin, and takes a deep eosin stain. This change extends throughout the cell. The nucleus undergoes karyorhexis. These altered cells stain with Weigert's fibrin stain. The cell is crowded aside and the space it formerly occupied becomes filled with

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phagocytes. By the disappearance of several adjacent liver cells, the cell clumps may attain a considerable size and conform to the lymphoid nodules of the older writers on typhoid fever.

To decide definitely whether this disintegration of the liver cells is due primarily to the presence of the phagocytes obstructing the capillaries, or whether they may not be attracted by the disintegrating liver cells is difficult. There is some evidence in favor of the latter view, as isolated liver cells undergoing necrosis in the manner described may be found, in whose neighborhood no phagocytes are to be seen. Since the giant cells [1] of the bone-marrow, which are many times larger than the phagocytes, are often found passing through the liver capillaries without producing any necrosis, the idea that the large size of the phagocytic cells precludes their passing through, and thus produces an obstruction of the capillaries, cannot be maintained.

Owing to their obstruction, the capillaries, which at first are rather narrow, become over distended and packed full of closely approximated red blood corpuscles, which frequently are shadow forms. From the pressure so produced, the trabeculæ of liver cells are reduced in many lobules to narrow bands not more than half their normal width. They commonly show fatty and other degenerative changes. In rabbits, small masses of bile pigment are often found in them. The endothelium of the capillaries is separated in many places from the liver trabeculæ by a serous exudate. Hemorrhage not infrequently takes place into these spaces. When phagocytic cell thrombi occur in neighboring capillaries a number of liver cells may be surrounded and cut off by their confluence. These enclosed cells undergo coagulation necrosis.

In addition, a variety of changes, such as usually accompany disturbed metabolism, but which are not specific enough to warrant description here, take place in the liver cells. A

[1] Smith notes the normal occurrence of the giant cells of the bone-marrow in the spleen of mice. Jour. Exper. Med., Vol. VI., p. 1. In all the spleens examined I have found them in considerable numbers.

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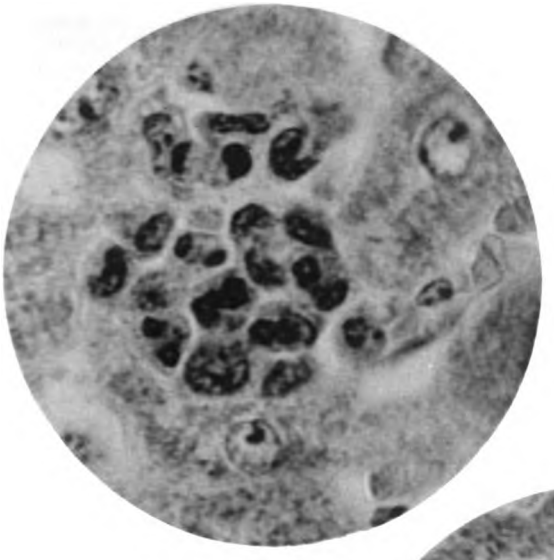

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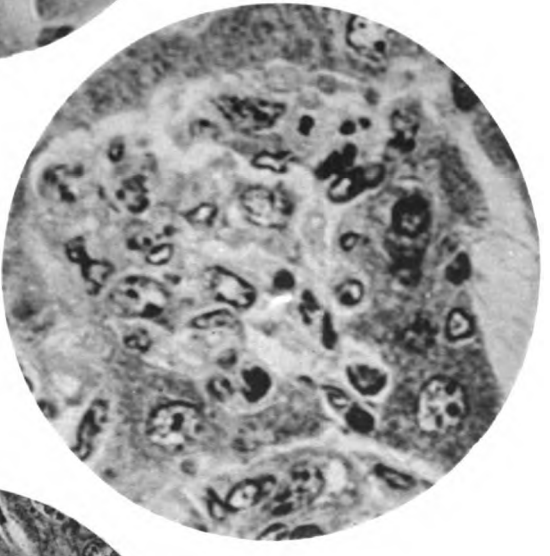

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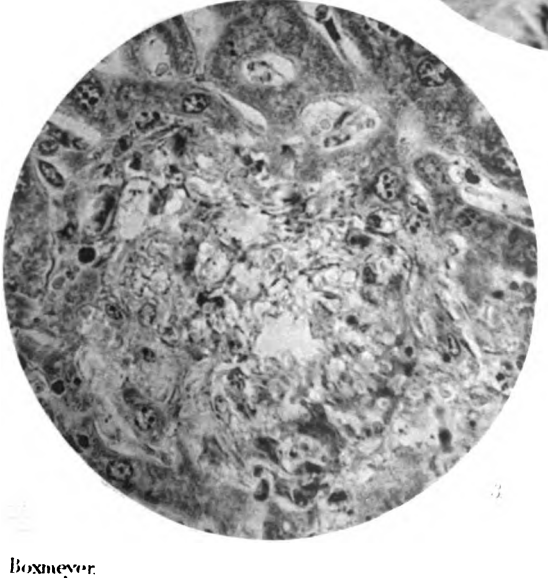

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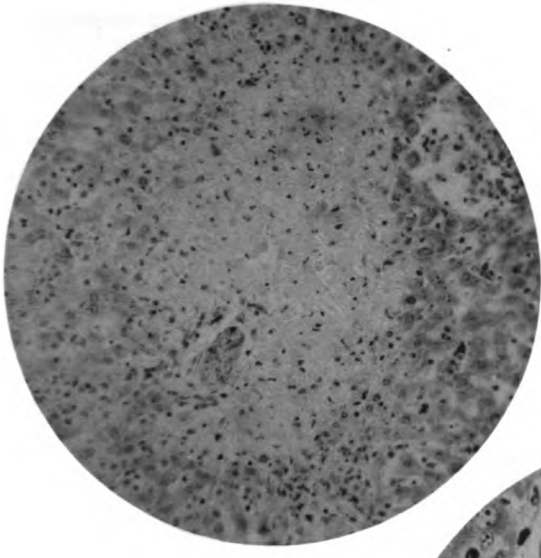

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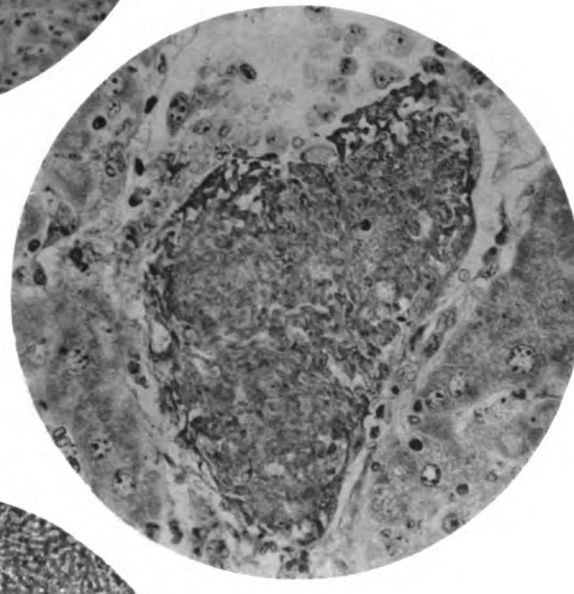

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more or less extensive fatty degeneration is frequently present.

From some experiments of Mallory's it appears that in guinea pigs, at least, clumps of phagocytes are either normally present in the liver capillaries, or may be produced by rough handling. In order to determine if such were the case with mice, I examined sections of the livers of several mice, both wild and tame. One had had an injection of sterile bouillon. The others had not been inoculated and were handled as little as possible.

In every case such cell clumps were found. But they were smaller, and present in much smaller numbers than in the inoculated animals. The degenerative changes, too, in them were much less marked.

While the formation of phagocytic cell thrombi is by no means specific for this disease, it is plainly seen that they are greatly augmented in number and size by it, and the degeneration proceeds much farther than ordinarily.

In some mice clumps composed for the most part of cells considerably larger than those already described are found in the liver capillaries. Many of these cells are filled with an amorphous yellowish-brown pigment (hemosiderin?). One or more shrunken and degenerated nuclei may be present. Some of the cells stain with eosin such a way as to suggest that they may be phagocytic cells filled with partially digested red blood corpuscles. Smaller cells of the same sort are also found.

In the spleens of mice and guinea pigs the phagocytic cell clumps attain, in certain cases, a relatively large size. They undergo coagulation necrosis, and show on the surface of the organ as yellowish-white patches. In the spleens of rabbits they have not been observed, and if present are masked by the great congestion of that organ in these animals.

The lesions so far described are essentially the same as those found by Mallory in typhoid, and agree fairly well with those found by Flexner in rabbits poisoned by abrin and ricin. Reed, by the injection of typhoid cultures into the mesenteric vein of rabbits, produced in their livers apparently

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the same sort of lesions, but he gives to them a quite different interpretation.

HYALIN THROMBI. — In addition to the lesions given above, we have others of quite different origin, the yellowish-white patches first mentioned. While the first sort affect, usually, only a small portion of the liver lobule, the second involve the greater portion of one or several lobules (Fig. 4). The surface lobules are the ones most often affected, the reason for which we shall see when we come to discuss their origin.[1]

In sections stained with eosin-methylene blue the necrotic areas stand out in sharp contrast to the normal tissue (Fig. 4). The liver cells, while retaining their shape, stain deeply with eosin. The basic staining particles present in the normal cell are absent, and the cytoplasm shows a finely reticular structure instead of the normal granular one. In these cells the bladder-like nucleus of the normal cell has shrunken to less than half its former size. At first it stains rather darkly, but as the shrinking continues it loses its power to stain with basic dyes, and can be distinguished from the cytoplasm only by its greater density and hyalin appearance. Later it disappears completely.

The endothelium of the capillaries of these areas does not undergo degeneration so soon as the liver cells, and it remains unaffected for some time. The capillaries may be empty, but are generally filled with phagocytes and blood corpuscles. Invasion of these areas by polymorphonuclear leucocytes occurs but rarely — a fact noted by Smith. Phagocytic thrombi are often included in them.

In mice it is on the fourth day after inoculation that we first find these necrotic areas, and then, also, for the first time, one sees hyalin thrombi in the veins. On the fifth day both thrombi and anemic areas are more numerous.

The thrombi occur in both the portal and hepatic veins.

[1] Dr. Smith tells me that foci of coagulation necrosis are most pronounced in the livers of rabbits inoculated with cultures recently isolated from swine. The power of the bacilli to produce these necroses appears to be lost, to a certain extent, by cultivation, and is not restored by passage through a series of rabbits. It is not possessed by varieties having a low degree of pathogenic power.

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They may be free or attached to the vessel walls, and not infrequently they lie just beneath the endothelial lining which has been raised by a serous exudate. In size and shape they vary greatly. Those in the larger vessels have a spherical or ovoid form, while those in the smaller are cylindrical and often extend some distance along their axis. In the capillaries they are present as cylindrical plugs. They attain their greatest size in the hepatic veins and there they are found in greatest number, yet they are not uncommon in the portal veins.

These thrombi occlude the larger veins partially, but the smaller ones often completely. To this obstruction of the smaller veins, the portal especially, the necrotic areas already described are due, as one may readily demonstrate, by means of serial sections. That the surface lobules should be most often and extensively affected is to be expected, since their blood supply is less complete, and in case of the obstruction of one of the portal vessels fewer chances of establishing a collateral blood supply remain than in the case of the more deeply lying lobules.

When the obstruction occurs in the portal veins or the capillaries leading from them, the relation of the thrombi to the areas of anemic necrosis may be readily traced; but when thrombi are found obstructing the central veins of the affected lobules only, as appears to be the case in rabbits, it is more difficult to understand. In the latter case the portal veins leading to these areas are distended with blood, as are also the capillaries. The central vein beyond the thrombi is empty of red corpuscles. The necrosis is confined chiefly to the center of the lobule, the liver cells immediately adjoining the portal veins remaining unaffected.

In the capillaries also thrombi occur, and they, too, give rise to anemic areas which undergo coagulation necrosis. Owing to the hyalin structure of these capillary thrombi and their property of staining with Weigert's fibrin stain, it is difficult in many cases to distinguish them from fibrin masses or the hyalin-degenerated cells, yet they can generally be shown to be of the same composition as those present in the veins.

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The thrombi (Fig. 5) are composed of a substance hyalin at the periphery and finely granular at the center. They are not homogeneous, but appear to be made up of a tangle of coarse cords among which a few phagocytes are caught. Fibrin threads cannot be demonstrated in them, yet may be deposited upon them and extend to the vessel walls. In the clefts at the periphery, normal blood corpuscles are often seen. Adhering to the thrombi are phagocytes, often in such numbers that they completely occlude the veins. The phagocytes are frequently bound together by fibrin.

These hyalin thrombi stain a violet-red color with eosin-methylene blue, and an orange or orange-red with picro-acid-fuchsin, in contrast to the lemon-yellow of the normal red blood corpuscles. With Weigert's fibrin stain they usually retain the stain, especially at the periphery. They also stain deeply with iron hematoxylin. In every case the periphery stains more deeply than the center. It appears that we have here to deal with the so-called hyalin thrombi.

Von Recklinghausen was the first to recognize them as of a class by themselves. He believed them to be derived from the leucocytes. Since then they have been described in many toxic states and infectious diseases by numerous writers. They have been found most frequently in the capillaries, in the form of hyalin casts. They have also been seen as balls and cylindrical masses in the smaller veins and arteries. Von Recklinghausen found them in spontaneous gangrene and ergotin poisoning, Kriege in the vessels of a rabbit's ear after freezing, Kaufman in corrosive sublimate poisoning, Ribbert observed them in rabbits poisoned with Staphylococcus pyogenes aureus, and Welch in the kidneys of cholera hogs. Mallory describes in the vessels of the intestine in typhoid fever what he calls "fibrin thrombi," but which are probably of this same nature. Klebs is of the opinion that these thrombi consist of coalesced and altered red-blood corpuscles. Ziegler states that they are formed from the colorless elements of the red blood corpuscles. Silberman, Werhofsky, and others also describe thrombi formed from more or less altered red corpuscles.

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While the consensus of opinion appears to be that thrombi of this sort owe their origin to red blood corpuscles, nevertheless much doubt concerning their true nature still remains.

In sections of liver hardened in absolute alcohol and stained with methylene blue one sees the thrombi as brown masses of various sizes and of the same color as the red corpuscles. The brown color is due to hemoglobin and suggests at once what blood elements compose them. In most cases, however, the outlines of the red corpuscles cannot be distinguished, and from the structure of these alone one gets absolutely no clew of their origin. Yet by examining a large number one can trace the steps in their formation.

In what may be considered the first stage one sees groups of corpuscles lying more closely together than the remainder of the corpuscles in the same vessel. They also stain somewhat differently from the others. Next one sees clumps in which the corpuscles at the center are fused into a homogeneous mass, while those at the periphery retain their normal form to a certain extent. The corpuscles show a tendency to unite into cords which form a tangle in the larger thrombi. In the completely-formed thrombi the shape of the corpuscles can no longer be distinguished, and the center becomes finely granular.

Preliminary to and during the fusion of the corpuscles to form thrombi they suffer a change in their chemical composition, which is shown by their abnormal staining reaction. That the thrombi are not composed of shadow corpuscles, as Ziegler states, is shown by their brown color in unstained sections; yet, that the corpuscles composing them differ from the normal ones is shown by their tendency to retain basic dyes, such as methylene blue and gentian violet. In sections treated with Weigert's fibrin stain by which the thrombi are usually stained, we often find lying near them in the same vessel a few red corpuseles that also retain the stain, though the remainder are all decolorized. Hence the presence of fibrin is not necessary to account for this staining peculiarity.

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The thrombi appear to acquire rapidly their final structure, for those found earliest in the disease are fully as far advanced as any found later. It is probable, although we find thrombi varying in size from those consisting of but a few corpuscles to those that would require many hundreds to give them their size, that most of them are of full size from the first and do not increase except by the adhesion of white cells to them and the deposition of fibrin upon them. Yet some of the larger appear to consist of several smaller ones more or less intimately united. Their formation does not occur wholly at one stage of the disease, but they continued to be formed from the fourth day after inoculation up to the time of death, immediately preceding which the process is probably most active, since we find at death thrombi in all stages of formation.

Stillmark has shown that ricin, when added in small quantities to defibrinated blood, causes an agglutination of the red corpuscles. Hellin has demonstrated a like action for abrin. Mitchell, Flexner, and others have made similar observations upon certain snake poisons. Experiments on the effects of introducing these poisons into the circulation have, however, not yielded such certain results. Still Werhovsky claims to have found thrombi formed by the fusion of red corpuscles in the capillaries of the liver of rabbits killed by abrin. Flexner failed to find any such thrombi in rabbits poisoned with abrin or ricin.

From the similarity of the action of ricin and abrin to many bacterial toxins, one could readily ascribe this clumping of the red corpuscles to form thrombi to the production by the bacteria of a toxin acting after the manner of the above poisons and producing agglutination of the corpuscles.[1]

Such a toxin might be produced directly by the bacillus

[1] Shortly before the completion of this manuscript, Flexner presented before the American Association of Pathologists and Bacteriologists, at Cleveland, a preliminary communication "On thrombi composed of agglutinated red blood corpuscles." He has produced such thrombi by the injection of agglutinating substances — ricin, ether, globucidal sera. He has found them in cases of typhoid, bronchopneumonia, and carbolic acid poisoning.

The writer had no knowledge of this paper until it appeared in the November number of the Journal of Medical Research, 1902, Vol. viii, No. 2.

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and set free either during its life or upon its dissolution, or indirectly by the action of the toxin upon the tissues, causing such a substance to be liberated from them upon their destruction.

The latter method would also explain the formation of such thrombi in other instances, freezing, burning, and poisoning by certain mineral substances, in all of which cases a destruction of tissue takes place. That the bacteria play an important part in the formation of the thrombi is shown by their being formed in greatest numbers late in the disease when the bacteria are present in the largest numbers.

That any agglutinin is present, there is, as yet, no positive evidence and quite a different line of experiments would have to be undertaken to demonstrate its presence. I hope to follow this line up later. The idea is merely suggested here, as it seems to afford a satisfactory explanation of the phenomena.

RELATION OF THE BACTERIA TO THE LESIONS. — Search for the bacteria in the liver was made mostly in sections. The culture method was used only in the examination of the control mice for bacteria other than hog cholera which might give rise to phagocytic thrombi.

Material for sections was hardened both in absolute alcohol and Zenker's fluid. The sections were stained either with alkaline methylene blue and decolorized with dilute acetic acid or with eosin methylene blue. Both methods gave good results.

In none of the control mice were bacteria found by either method. In the section of the livers of the mice of the series killed at different intervals after inoculation, no bacteria which could certainly be identified as hog cholera bacilli were found on microscopic examination. Mice 11, 16, and 18 showed a few bacteria of doubtful identity, the remainder none.

Bacteria were found without exception in sections of the livers of all the mice that had been allowed to die of the disease. In most instances they were present in great

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numbers, but in others a few only were found after long and careful searching. In mouse 26, which was chloroformed while dying, bacteria were found in considerable numbers. The greatest number of bacteria was found in the livers of those mice which had lain longest before fixation. In these cases a post mortem multiplication probably took place. The bacteria were always found in clumps, some quite large, others very small. Bacteria were also constantly found in sections of the livers of rabbits which succumbed to injections of hog-cholera bacilli.

It is in the anemic areas that the bacteria are found in greatest numbers. They lie either in the capillaries or in the necrotic liver cells. They are present in the phagocytic cell thrombi and in capillaries surrounded by perfectly normal liver tissue.

The bacteria were quite often found in isolated necrotic liver cells. In many cases they appeared to be but the remains of bacteria, as they stain but feebly. Earlier in the disease, when these cells were first observed, no bacteria were found in them, so it is probable that the bacteria enter these cells only after the death of the latter, and are not the cause of their degeneration. In no other cases could any direct relation of the bacteria to the lesions present be demonstrated, and their connection here is exceedingly doubtful.

SUMMARY.

1. Two sorts of lesions are present. One is due to the plugging of the capillaries by large mononuclear cells, and probably, also, to the direct action of a toxin upon the liver cells. The other is due to the plugging of the smaller veins and capillaries by hyalin thrombi.

2. Early in the disease there is a marked proliferation of the endothelial cells of the blood and lymph vessels and serous surfaces. This proliferation is especially active in the lymph spaces of the spleen. The cells produced have but a low vitality, and possess, to a certain degree, phagocytic powers.

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3. The hyalin thrombi are composed of slightly altered and coalesced red blood corpuscles.

4. No direct relation of the bacteria to the lesions could be shown.

(The writer wishes to express his thanks to Dr. Smith for the help and suggestions given him in this study.)

May, 1902.

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DESCRIPTION OF PLATES.

The figures are reproductions of photo-micrographs made by Mr. L. S. Brown, with the kind permission of Dr. J. H Wright, of the Clinico Pathological Laboratory of the Massachusetts General Hospital.

All sections are from material fixed in Zenker's fluid, and stained with eosin polychrome methylene blue.

PLATE X.

Fig. 1. Clump of young, large mononuclear cells obstructing a capillary. They have displaced one or two liver cells, whose remains show as a light band near the mitotic figure. Liver of mouse No. 18, x 1000.

Fig. 2. Clump of large mononuclear cells lying in a capillary space. They have lost the power of staining with basic dyes, and show other degenerative changes. Necrotic liver cells at the periphery. A few polymorphonuclear cells among the phagocytes. Liver of mouse 17, x 1000.

Fig. 3. Area formerly occupied by phagocytic cell clumps. Now filled by a network of fibrin containing cell remains. Liver of mouse 5, x 250.

PLATE XI.

Fig. 4. Area of coagulation necrosis due to thrombosis of the portal vein by a hyalin thrombus. Liver of mouse 2, x 50.

Fig. 5. Parietal thrombus of the hepatic vein. Thrombus composed of agglutinated and partially fused red corpuscles. Many phagocytic cells in the vein. Liver of mouse 2, x 250.

Fig. 6. Area of coagulation necrosis in the liver of a rabbit due to thrombosis. A small hyalin thrombus in a hepatic vein, x 20.

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Reprinted from the American Journal of Physiology.
VOL. XI. — APRIL 1, 1904. — NO. I.

STUDIES ON THE "PARADOXICAL" PUPIL-DILATATION CAUSED BY ADRENALIN. I. — THE EFFECT OF SUBCUTANEOUS INJECTIONS AND INSTILLATIONS OF ADRENALIN UPON THE PUPILS OF RABBITS.

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[*From the Rockefeller Institute for Medical Research.*]

IN our studies of the effect of intravenous injections of adrenalin upon the blood-vessels of rabbits' ears,[2] we made the observation that section of the cervical sympathetic nerve changes this effect in some respects: the constriction of the vessels of the corresponding ear sets in later, develops gradually, reaches the maximum later, and lasts incomparably longer than in the ear of a normal animal. In further studies upon subcutaneous injections,[3] the influence of the section of the sympathetic nerve was still more marked. While in a normal animal a moderate dose of adrenalin has either no effect, or the effect is rather a marked dilatation of the blood-vessels, a subcutaneous injection of such a dose of adrenalin into a rabbit in which the sympathetic nerve is cut or resected, effects invariably a long-lasting constriction of the blood-vessels of the ears.

Section of the sympathetic nerve, then, has a distinctly modifying influence upon the vasomotor effect of the extract of the suprarenal gland, at least as far as the ear-vessels are concerned. The question presented itself, whether section of the sympathetic nerve could also influence the effects of the suprarenal extract upon other organs which stand under its control. Such an organ is the pupil. Lewandowsky[4] discovered that intravenous injection of suprarenal extract causes a dilatation of the pupil. This was confirmed by Boruttau[5] and by

[1] Research scholar of the Rockefeller Institute for medical research.
[2] MELTZER and MELTZER: This journal, 1903, ix, p. 147.
[3] MELTZER and MELTZER: This journal, 1903, ix, p. 252.
[4] LEWANDOWSKY: Archiv für Physiologie, 1899, p. 360.
[5] BORUTTAU: Archiv für die gesammte Physiologie, 1899, lxxxviii, p. 112.

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Langley.[1] The effect is very brief, it lasts less than a minute. It is distinct in cats, is less pronounced in rabbits. All agree that a subcutaneous injection has no effect, and this is explained by the assumption that the extract, before it reaches the blood, is oxidized in the lymph spaces.

In extensive experience with subcutaneous injection of adrenalin in rabbits, we can confirm the observation that in normal animals the pupils are never affected by an injection of even a large dose, provided it does not cause asphyxia of the animal. It is different, however, with operated animals. In our studies of the effect of subcutaneous injections of adrenalin upon the vasomotor condition of the ear-vessels, during which, in a large number of rabbits, the sympathetic nerve was cut, or the superior cervical ganglion was removed, it was found that in many cases the pupil became dilated on the operated side after a subcutaneous injection; but the effect seemed to be inconstant. An analysis of our notes brought to light, however, that the phenomenon was observed only in those animals in which the ganglion was removed, and when the injection was made at least twenty-four hours after its removal. To test this point we instituted a series of experiments on rabbits in which the relations of the excision of the ganglion and the cutting of the sympathetic nerve to the effect upon the pupil of a subcutaneous injection of adrenalin were studied systematically. The results we now obtained were constant and perfectly uniform. They are, briefly stated, as follows: —

After section of the sympathetic nerve, a subcutaneous injection of even a large dose of adrenalin has no effect on the pupil. This can be said to be the rule. In a very few exceptions the injection seemed to cause a slight dilatation. It was, however, under all circumstances very slight, lasting only a few seconds, and in fact, we were never very sure of its occurrence.

When, however, the superior cervical ganglion was excised, the effect of a subcutaneous injection of adrenalin upon the pupil was a very striking one. Even after the injection of such a moderate dose as 0.6 c.c., the pupil on the side on which the ganglion was removed became dilated *ad maximum*. It is safer, however, to employ a somewhat larger dose, — about 1.0 or 1.2 c.c. of adrenalin chloride (1 : 1000). In ten or fifteen minutes the pupil then becomes very dilated, and remains so for at least one hour, and very often for

[1] LANGLEY: Journal of physiology, 1901–1902, xxvii, p. 237.

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more than even two hours. In some cases the dilatation begins to show three to four minutes after the injection.

When the dilatation caused by adrenalin is *ad maximum*, the dilated pupil does not react to light.

When previous to the administration of adrenalin the pupils were made myotic by means of instillation of eserin, the subcutaneous injection of adrenalin caused, nevertheless, an *ad maximum* dilatation of the pupil on the operated side, while on the normal side the myosis was not affected in the least. Adrenalin apparently overcomes completely the effect of eserin.

The dilating effect of adrenalin upon the pupil can be seen only when the subcutaneous injection is made at least twenty-four hours after the removal of the ganglion. Injections made at an earlier period exercise apparently no effect upon the pupil, — while the vaso-constricting effect upon the ear-vessels of the operated side is very distinct, even if the injection be made soon after the operation.

The effect upon the pupil takes place only when the entire ganglion is removed. Section of all the connecting branches except the upper one, or removal of the lower two-thirds of the ganglion, does not lead to a response of the pupil by dilatation after a subcutaneous injection of adrenalin. In tearing out the ganglion in earlier experiments, we met with a few failures. On reopening the wound it was found that the removal was incomplete. The subsequent removal of the remaining portion brought out the usual result.

We have also studied the effect upon the pupil of instillation of adrenalin into the conjunctival sac after removal of the ganglion. Radziewsky[1] was the first to state that instillation of suprarenal extract exerts no effect upon the pupil. This was confirmed by Lewandowsky[2] and Boruttau.[3] From our studies we can confirm the observation that instillation does not affect the pupil of a normal rabbit. Neither does it cause any change in the pupil even after the sympathetic nerve is cut. However, twenty-four hours after complete excision of the superior cervical ganglion, instillation of adrenalin causes a dilatation of the pupil on the operated side. The degree of the dilatation depends upon the quantity instilled. After four or five instillations

[1] RADZIEWSKY: Berliner klinische Wochenschrift, 1898, p. 572.
[2] LEWANDOWSKY: *Loc. cit.*
[3] BORUTTAU: *Loc. cit.*

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of two drops at a time at intervals of two or three minutes, the dilatation can be *ad maximum*. The dilatation lasts also for some time —several hours; usually, however, it disappears sooner than after a subcutaneous injection. The myotic effect of eserin is also overcome by instillation of adrenalin. We have never observed constitutional effects from instillation; for instance, we have never seen a constriction of the ear-vessels taking place after otherwise effective instillations of adrenalin.

The difference in the effect between section or resection of the sympathetic nerve and excision of the superior cervical ganglion is brought out best when the ganglion is removed on one side and the sympathetic nerve cut on the other side of the same animal. After a subcutaneous injection of a medium dose of adrenalin, there is then no difference in the condition of the ear-vessels on the two sides, both ears look equally pale; but there is a striking contrast in the pupils, the pupil on the side without the ganglion is widely dilated, while the one on the other side remains more contracted than normal, on account of the section of the sympathetic nerve.

We have frequently employed the following method to bring out some of the above-described results in a striking manner. The hind leg of an operated rabbit was tightly ligated above the knee and a medium dose of adrenalin injected into the leg peripheral to the ligature. When after some time the ligature was removed, the pupil on the side on which the ganglion was removed became widely dilated in less than half a minute, while on the normal side or the side on which only the sympathetic nerve was cut the pupil remained unchanged. It required some experience to attain the proper degree of tightness of the ligature. In a rabbit it is quite difficult to apply a single ligature tight enough so that none of the adrenalin shall escape under the ligature. We then often see a slight dilatation of the pupil (and constriction of the ear-vessels) taking place before the ligature is removed. A double ligature will safely prevent the escape. But then the ligature has to be removed in less than an hour; otherwise the absorption becomes impaired and also frequently leads to gangrene of the leg. However, after some experience it is not difficult to find a proper degree of tightness (best two ligatures of moderate tightness) which will prevent any escape and which at the same time will not permanently impair absorption. In such a case, the ligature can be kept for two hours and even longer, and after its removal the effect of adrenalin is a striking and nearly an instan-

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taneous one. We shall discuss later the significance of such an experiment.

Our experience with the effects of intravenous injections of adrenalin on the pupil, is still too scanty to permit any detailed statement; we gathered it only incidentally in experiments carried on for other purposes. As far as our experience goes, intravenous injections showed the same conditions as were observed in the subcutaneous injections and instillations, *i. e.*, on the side on which the ganglion was removed the pupil also became dilated after an intravenous injection, and the dilatation lasted for quite a long time; whereas on the normal side or on the side on which the sympathetic nerve was cut, the dilatation was comparatively insignificant and lasted only a few seconds. We may, however, come back to this subject in a later communication.

We shall illustrate the above-stated results by a few protocols of experiments. They are greatly abbreviated, as many of these experiments were utilized also for a study of the vasomotor phenomena in the ears.

Experiment 53. Dec. 1, 1902. — Large brown rabbit.[1] Right ganglion torn out, . . . left side normal. . . .

Dec. 2. — . . . No difference in size of the two pupils.

Dec. 3. — 4.00 P. M.: Subcutaneous injection of 1.5 c.c. of adrenalin (1 : 1000), right pupil much dilated, no noticeable effect upon left. 5.30, 7.00, and 9.00 P. M.: No change.

Dec. 4. — 10.00 A. M.: Both pupils about equal. 2.45 P. M.: Injected subcutaneously 1.5 c.c. 2.50: Right pupil large. . . . 3.10: Right pupil very wide. . . . 4.40 and 6.00 P. M.: Right pupil wider than left. 9.00 P. M.: Right pupil same size as left.

Dec. 12. — Right pupil smaller than left.

Dec. 18. — Right pupil smaller than left. Subcutaneous injection of 1.5 c.c. . . . Right pupil much dilated.

Dec. 23. — 8.00 P. M.: Subcutaneous injection of 0.6 c.c. In a few minutes right pupil wide. 9.00 P. M.: Right pupil smaller again, but still wider than before injection.

Dec. 24. — Hind leg ligated, injected below ligature 1.2 c.c. of adrenalin, . . . "ligature taken off in five to seven minutes, the right ear became pale immediately, the left ear still full; the right pupil very wide."

In this experiment repeated injections invariably brought out a prompt effect, — even a dose of only 0.6 c.c. caused an unmistakable

[1] All animals were narcotized during operation.

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dilatation. With doses of 1.5 c.c. the dilatation lasted at least three hours.

Experiment 61. — Small gray rabbit. . . .

Dec. 9. — Cervicals operated.

Dec. 16. — Wound reopened and left ganglion removed. . . . 8.30 P. M.: Left pupil smaller. Injected subcutaneously 1.5 c.c. Right ear, no change; left ear pales gradually; no effect on eyes.

Dec. 17. — 8.30 P. M.: . . . left pupil a trifle wider than right. Injected subcutaneously 1.5 c.c., . . . in a few minutes left pupil wider than right.

Experiment 62. *Dec. 10.* — Right sympathetic cut.

Dec. 16. — Right pupil a trifle smaller than left. 11.45 A. M.: Injected subcutaneously 1.5 c.c. . . . No effect on eyes. 4.15 P. M.: Right pupil a trifle smaller. Wound reopened and right ganglion torn out. . . . 6.00 P. M.: Right pupil smaller. . . . 8.00 P. M.: Injected 1.5 c.c.; right ear becomes pale; . . . no effect on eyes; . . . a second injection: no effect on pupils.

Dec. 17. — 6.00 P. M.: Pupils about equal. Injected subcutaneously 1.5 c.c. . . . In three minutes the right pupil begins to dilate, in fifteen minutes very wide. . . . 9.45: Right pupil much wider than left.

Dec. 23. — 6.00 P. M.: Hind leg tightly tied with Esmarch bandage; injected into distal end 2.0 c.c.; toxic symptoms while bandage still on; bandage removed; . . . right pupil very wide. 9.00 P. M.: Right pupil wide.

Dec. 24. — Injected subcutaneously 1.00 c.c. Right pupil becomes wide.

Jan. 14, 1903. — 5.30 P. M.: Leg very firmly tied. No effect noticed. Ligature removed at 7.00 P. M. In two minutes pupil very wide.

In this experiment section of the sympathetic had no effect; but twenty-four hours after subsequent removal of the ganglion, the effect was invariably positive. It shows also that even a firmly applied Esmarch bandage permitted the escape of the adrenalin.

Experiment 67. *Jan. 13, 1903.* — 5.30 P. M.: Right ganglion removed; . . . right pupil much smaller than left.

Jan. 14. — Evening. Instilled into both conjunctival sacs adrenalin. In about five minutes the right pupil began to dilate slowly. . . . In three hours still widely dilated. No effect on left pupil.

Experiment 70. *Jan. 27, 1903.* — Large rabbit. Right sympathetic resected; after ten minutes right pupil smaller than left.

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Jan. 30. — Injected subcutaneously 1.2 c.c. adrenalin; . . . effect on ear-vessels but not on pupils.

Jan. 31. — 11.30 A. M.: Left ganglion removed, . . . pupils about equal, left conjunctiva injected. 6.00 P. M.: Instilled adrenalin into both conjunctival sacs, no result.

Feb. 1. — 11.00 A. M.: Left pupil somewhat wider than right. Instilled adrenalin into both conjunctival sacs; right pupil, no change, left dilates slowly; after three-quarters of an hour, well dilated; no reaction to light.

Feb. 2. — Leg tied with double ligature; injected 1.5 c.c. adrenalin peripherally to ligature; apparently no escape; ligature removed after two and a half hours; twenty-five seconds later left pupil widely dilated; no change in right pupil.

Experiment 77. March 2. — Right ganglion removed, left sympathetic resected.

March 4. — Instilled adrenalin into both conjunctival sacs. In two minutes right pupil rapidly dilating; in ten minutes dilated ad maximum; no change in left pupil.

These few protocols are sufficient to illustrate the essential points of our statements. But we have made quite a large number of such experiments, and all gave uniformly the same results. In nearly a dozen instances the experiments were demonstrated to interested observers without a single failure. The previous use of eserin brings out the main phenomenon in a striking way.

The longest period during which we had operated animals under observation was three and one-half months. The effect of a subcutaneous injection was at the end of this period as pronounced as in the first few days after operation. The effect of instillations seemed somewhat diminished, the dilatations did not reach the maximum, and returned to normal sooner than before. However, these observations have not been numerous enough as yet to permit positive conclusions to be drawn.

Our protocols of the above-mentioned series of experiments contain a few notes with reference to the effects of section of the sympathetic nerve and removal of the ganglion upon the condition of the pupil, which may deserve to be recorded.

In a few cases the ganglion was removed a few days after the sympathetic had been resected. In none of these cases was the constriction of the pupil increased by the removal of the ganglion. Furthermore, when the sympathetic nerve was cut on one side, and at

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the same time the ganglion was removed on the other side, the constriction of both pupils was, in our experiments, the same. Budge,[1] Langendorff,[2] and others state that on the first day after the operation, the pupil on the side on which the ganglion was removed is more constricted than on the side on which the sympathetic was cut, and the conclusion is therefore drawn that besides the dilating tonus, kept up by the fibres of the sympathetic nerve, an additional dilating tonus emanates from the cells of the superior cervical ganglion. Kowalewsky[3] and other writers, however, could not confirm this statement. Our own restricted experience seems to agree with that of the latter authors.

In most of the experiments on the removal of the ganglion, we find that on the day after the removal, the pupil on the operated side was wider again, — being mostly "about equal" to the pupil of the other side, if the latter was normal, or "a trifle wider" when the sympathetic was cut on the other side. Similar observations have been already recorded by Budge,[4] and were subsequently observed by a number of other writers. We find in our notes, however, that in some cases, from the seventh day on after the operation, the pupil on the ganglion side was smaller again than on the normal side (see above, Experiment 53). In others the conditions seemed to vary during the entire period of observation, sometimes the pupil on the ganglion side being equal to that of the sympathetic side, and at other times being a trifle larger, without there being any visible reason for the variations.

We have never noticed that excitement of the animal favored a dilatation of the pupil on the ganglion side. Neither have we observed that the usual state of even deep anæsthesia (we employed only ether) brought out a greater dilatation of the pupil on the ganglion side. In two cases, however, in which the very deep anæsthesia brought on asphyxia of the rabbits, the pupil on the ganglion side was noticed to be visibly wider than on the sympathetic side; the latter also became somewhat dilated.

In a number of protocols of experiments in which the ganglion was removed, it was noted that the conjunctiva on the operated side was

[1] BUDGE: Ueber die Bewegung der Iris, Braunschweig, 1855, p. 125.

[2] LANGENDORFF: Klinische Monatsblätter für Augenheilkunde, 1900, xxxviii, p. 129 and p. 823.

[3] KOWALEWSKY: Archives slaves de biologie, 1886, i, p. 592.

[4] BUDGE: *Loc. cit.*

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injected. There is no such remark in any case in which the sympathetic was cut.

In three rabbits the sympathetic nerves were stimulated low down in the neck many weeks after the operation. In two rabbits in which the ganglion was removed on one side, the corresponding sympathetic nerves were stimulated forty-five days after the operation. With very strong induction currents there was a moderate constriction of the ear-vessels; no effect upon the pupil. In a third rabbit in which the ganglion was removed on one side, and the sympathetic nerve was resected on the other side, the sympathetic nerves were stimulated seventy-one days after the operation. There was a fair constriction of the blood-vessels on the ganglion side, with a moderate current (300 units, Kronecker's model), while there was no effect at all either on the blood-vessels or on the pupil on the side on which the sympathetic nerve was resected, even when stimulated with very strong currents (3000 units and more). On this side apparently no regeneration took place even after seventy-one days.

We shall not enter into any discussion of the observations we have just recorded. They are not sufficient in number and were gathered only incidentally to our chief studies. The discussion of the latter will be carried out in a subsequent paper.

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STUDIES ON THE "PARADOXICAL" PUPIL-DILATATION CAUSED BY ADRENALIN. II. — ON THE INFLUENCE OF SUBCUTANEOUS INJECTIONS OF ADRENALIN UPON THE EYES OF CATS AFTER REMOVAL OF THE SUPERIOR CERVICAL GANGLION.

By S. J. MELTZER.

[*From the Hallerianum in Bern.*]

BESIDES its influence upon the pupil, the cervical sympathetic nerve controls also the width of the palpebral fissure, the movements of the nictitant membrane and the position of the bulbus. The influences upon the latter phenomena are indistinct in rabbits, but are outspoken in cats. The studies in the foregoing paper were made on rabbits, and were therefore restricted to the influence of adrenalin upon the pupil. Last summer, while enjoying the unlimited hospitality of the physiological laboratory in Bern (thanks to the kindness of Professor Kronecker), I have made some observations also on the effect of adrenalin upon the eyes of cats. The experiments were made chiefly with subcutaneous injections. The results obtained are, briefly stated, as follows.

Subcutaneous injections of even large doses of adrenalin have no effect upon the eyes of normal cats. When the sympathetic nerve (or more often the vago-sympathetic) was cut on one side, the pupil on the corresponding side became smaller, the palpebral fissure narrower, and the nictitant membrane covered a good part of the eye. (Of the position of the bulbus I was never sure enough, and can therefore make no positive statement with regard to it.) When now one c.c. or more of adrenalin was injected subcutaneously, a retraction of the nictitant membrane very soon followed; this occurred even if the injection was made soon after the operation. But at no time and with no dose of adrenalin could the condition of the pupils or the palpebral fissure be affected by a subcutaneous injection.

However, if the superior cervical ganglion was removed, a subcutaneous injection of adrenalin caused a distinct widening of the pal-

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pebral fissure and a complete dilatation of the pupil. This effect could be obtained only when the drug was injected at least 48 hours after the operation (24 hours in rabbits). At least 1.5 c.c. was required to bring out a distinct effect, even in a small and young animal. Older and larger animals required larger doses. The larger the dose, the sooner the phenomena appeared, and the longer they lasted, — the pupil sometimes remaining dilated for six hours and longer. Usually there was immediately after the injection a distinct dilatation of the pupil on the operated side, which, however, disappeared soon again, but reappeared in 15 to 40 minutes, according to the injected dose of adrenalin, to remain then unchanged for hours. The primary dilatation is probably due to the excitement of the animal, or to the pain produced by the injection; since both can, as we now know, bring on the so-called paradoxical dilatation of the pupil. I shall state, however, that by injection of water I could never bring on this primary dilatation of the pupil.

In no instance, even if the pupil was ever so dilated, was the reaction to light lost, or perhaps not even perceptibly affected. (In rabbits, as we have stated above, with the dilatation of the pupil there was also always an impairment or complete loss of the reaction to light.) However, the largest dose of adrenalin which I have ever used subcutaneously on operated cats was 3 c.c., and it is possible that a larger dose might indeed affect also the reaction. (I have avoided larger doses in order not to risk the life of the animal. It is worth mentioning, however, that with the exception perhaps of an occasional diarrhœa, in no case were the cats affected by even a dose of 3 c.c.; while even in a larger rabbit a dose of 2 c.c. would cause a marked paresis and general prostration.)

When the sympathetic nerve was cut on one side, and the ganglion removed on the other side, the contrast between the pupils and the palpebral fissures of the two sides was very striking. The retraction of the nictitant membrane occurred on both sides, but it seemed that on the ganglion side the retraction was more marked and lasted longer than on the sympathetic side. But even on the ganglion side the retraction gave way sooner than the other phenomena.

The described phenomena could be produced at will as long as the animals were kept alive, that is for about six weeks.

I have not been successful with instillations of adrenalin into the eyes of operated cats, on account of the protruding nictitant membrane which would sweep over the entire eye, as soon as the adrenalin

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came in contact with it, thus removing the adrenalin. But I must admit that I have not been persistent enough in my attempts.

I may perhaps put on record that in two instances I made an operated cat drink milk to which 6 c.c. of adrenalin had been added. About an hour after the partaking of the milk, the pupil on the ganglion side began to dilate. The dilatation lasted for a few hours, but was never considerable.

In one operated cat which was kept under a moderate ether anæsthesia, and in which there was no perceptible difference between the two pupils, an intravenous injection of 1 c.c. of adrenalin brought on a maximum dilatation of the pupil on the ganglion side which lasted for one hour. At the end of that time the cat was killed, but the dilatation of the pupil persisted for two hours longer.

Like Langendorff,[1] I have also observed that in a deeply etherized cat the pupil on the "ganglion side" becomes greatly dilated, while on the "sympathetic side" the pupil remains unchanged. This occurred only when the deep anæsthesia was carried on and completed under a glass bell, in the same manner as was stated by Langendorff. By this method, however, we have not only anæsthesia by ether, but also asphyxia by carbon dioxide. When, however, in an early stage of the anæsthesia the cat was quickly removed from under the bell and fastened on a board, and the ether administered in free air, the animal was under a fairly good anæsthesia without showing any marked difference between the two pupils.

[1] LANGENDORFF: *Loc. cit.*

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STUDIES ON THE "PARADOXICAL" PUPIL-DILATATION CAUSED BY ADRENALIN. III.—A DISCUSSION OF THE NATURE OF THE PARADOXICAL PUPIL-DILATATION CAUSED BY ADRENALIN.

By S. J. MELTZER and CLARA MELTZER AUER.[1]

[*From the Rockefeller Institute for Medical Research.*]

IN the foregoing reports it has been shown that subcutaneous injections (or instillations) of adrenalin have no effect upon the normal pupil nor upon a pupil whose sympathetic nerve was cut or resected. But if the superior cervical ganglion was removed, adrenalin invariably caused a strong and long-lasting dilatation of the corresponding pupil. The presence of the ganglion then prevents the dilating effect of adrenalin upon the pupil; by its excision something is removed which is normally an obstacle to the dilatation. It has long been known that when the sympathetic nerve is cut, the pupil becomes smaller. The generally accepted interpretation of this phenomenon is that the nerve fibres of the sympathetic are continually carrying mydriatic impulses to the pupil, which are discontinued by section of the nerve. It would seem to be the same logical interpretation to state that by the excision of the ganglion certain normal myotic impulses are removed. But we would then have to assume that the sympathetic nerve carries to the pupil from the central nervous system mydriatic impulses, and that the superior cervical ganglion sends myotic impulses through the postganglionic fibres. In other words, the ganglion generates and sends to the peripheral tissues impulses of its own, which in their character are just opposite to those sent by the central nervous system through the sympathetic nerve fibres.

However, the conception of the cervical sympathetic system as a conveyer of mydriatic impulses to the pupil is more than sixty years old and is deeply rooted. The early and oft expressed claim that the

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superior cervical ganglion sends up impulses of its own had reference only to such impulses as were exactly of the same character as those carried by the sympathetic nerve, — additional mydriatic impulses. When, therefore, facts became known showing the occurrence of the dilatation of the pupil after removal of the ganglion, these facts did not give rise to the logical interpretation that the ganglion might normally send myotic impulses; the phenomena were simply dubbed "paradoxical."

The term "paradoxical pupil-dilatation" is of very recent origin. It was introduced a few years ago by Langendorff[1] to designate a group of dilatations of the pupil occurring under certain conditions after removal of the superior cervical ganglion. To this group belongs in the first place also the old observation of Budge, that when the sympathetic nerve is cut on one side below the superior ganglion, and on the other side the postganglionic fibres are cut (thus excluding the influence of the ganglion), a day or two after the operation the pupil on the "ganglion side" is a little wider than the one on the sympathetic side. Of the other conditions which favor the appearance of the paradoxical pupil-dilatation we have to mention deep anæsthesia by chloroform, ether, or chloral, asphyxia, and excitement of the animal. If the ganglion were removed on one side, and the sympathetic nerve cut on the other side, these conditions would cause a wide dilatation of the pupil on the "ganglion side," with no effect upon the pupil on the "sympathetic side." It was also observed that curare had the same effect.[2]

Besides Langendorff, who studied the phenomena carefully, and who deserves special credit for having directed attention to them, the subject was investigated by many observers before him. Langendorff himself mentions as his forerunners, Budge,[3] Surminsky,[4] Tuwim,[5] and Schiff.[6] Besides these authors, the phenomena were

[1] LANGENDORFF: *Loc. cit.*

[2] It is, however, a question whether in the cases of anæsthesia as well as of curare the paradoxical mydriasis is not due to the concomitant asphyxia. In the above-described observations of one of us upon cats, anæsthesia without asphyxia never brought out the paradoxical pupil-dilatation. The same was observed by H. K. Anderson, who is of the opinion that the effect of anæsthesia "is not direct, but indirect by the anæsthesia it causes."

[3] BUDGE: *Loc. cit.*

[4] SURMINSKY: Zeitschrift für rationelle Medicin, 1869, xxxvi, p. 231.

[5] TUWIM: Archiv für die gesammte Physiologie, 1881, xxiv, p. 127.

[6] SCHIFF: Gesammelte Beiträge, 1896, iii, p. 107.

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carefully studied by Kowalewsky,[1] Braunstein[2] and Roebroeck.[3] Since the publication of Langendorff's paper, the subject was studied and discussed by Levinsohn,[4] Lewandowsky[5] and H. K. Anderson.[6]

We should add that the conditions which bring out the paradoxical pupil-dilatation cause also a widening of the palpebral fissure and a retraction of the nictitant membrane.

The paradoxical pupil-dilatation following the injection or instillation of adrenalin, which we have described above, is in most respects similar to the phenomenon observed under the other conditions. It cannot be due, however, to pain or excitement of the animal caused by the injection, since in all cases the dilatation takes place long after the injection; and it cannot be due to asphyxia, since it occurs after the use of such small doses as produce no ill effect whatever upon the animal; and since it is also produced by instillations which exert only a local influence. The effect is apparently due to the specific action of adrenalin upon the concerned tissues. But what is the nature of this action, and which tissues are affected? What is the mechanism of this mydriasis? For the interpretation of the paradoxical pupil-dilatation consequent upon the causes enumerated above (anæsthesia, etc.), there are a number of theories. Budge assumes that the total abolition of the tonus of the dilator pupillæ, which is accomplished by the removal of the ganglion, leads finally also to a relaxation of the sphincter, — a sort of atrophy by disuse. This theory was also accepted by Tuwim, and is at present advocated by Levinsohn. Surminsky assumed that these dilatations are caused by constrictions of the blood-vessels of the iris. Braunstein is of the opinion that the dilatations are caused by a cortical inhibition of the sphincter. Langendorff and also Roebroeck assume that, after the removal of the ganglion, the dilator is more or less in a state of contracture kept up by the processes within the degenerating post-ganglionic nerve fibres. Kowalewsky assumes that the ganglion sends

[1] KOWALEWSKY: *Loc. cit.*

[2] BRAUNSTEIN: Zur Lehre von der Innervation der Pupillenbewegung, Wiesbaden, 1894.

[3] ROEBROECK: Het Ganglion Supremum Colli Nervi Sympathici, Utrecht, van Boekhoven, 1895.

[4] LEVINSOHN: Klinische Monatsblätter für Augenheilkunde, 1900, xxxviii, p. 625; Archiv für Ophthalmologie, 1902, lv, p. 153.

[5] LEWANDOWSKY: Sitzungsberichte der Königlich-Preussischen Akademie der Wissenschaften, 1900, p. 1136; Archiv für Physiologie, 1903, p. 367.

[6] H. K. ANDERSON: Journal of physiology, 1903, xxx, p. 290.

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exciting as well as inhibiting nerve fibres to the dilator. Lewandowsky finally is of the opinion that the dilatation is due to an increased excitability of the dilator muscle owing to the loss of an inhibitory influence exercised by the ganglion over that muscle.

In stating our views as to the nature and course of the paradoxical state in question, we shall restrict our discussion to the pupil-dilatation caused by adrenalin. Neither shall we enter into a general discussion of the availability of the above-quoted theories for our special case. We shall try to state our interpretation of the phenomena observed by us without attempting to controvert the views promulgated by others.

There is, however, one of the older interpretations which seems to have been definitely disposed of by our present-day writers, but which, it seems to us, requires a discussion in our special experience. It is the theory that the pupil-dilatations are caused by a contraction of the blood-vessels. As the constriction of the blood-vessels is the chief characteristic of adrenalin, we have indeed to investigate whether the dilatation of the pupil is not simply due to this constriction.

When Lewandowsky[1] first discovered the brief dilatation of the pupil after an intravenous injection of the suprarenal extract, he already touched upon the question whether it be not due to the vasoconstriction of the vessels of the iris, but dismissed this suggestion by the remark that the dilatation is too great to be caused by the constriction of the vessels. Lewandowsky had apparently in mind the older view of the dilatation of the pupil being caused mechanically by the constriction of the blood-vessels. But with adrenalin there is another side to this question. The constriction of the vessels is so strong as to cause local anæmia or local asphyxia, and the question can be raised indeed whether the pupil-dilatation is not caused indirectly by the local asphyxia. The pupil-dilatation which we observed to occur after administration of adrenalin would thus be simply another variety of paradoxical mydriasis due to asphyxia. This might appear the more plausible since we know that after the removal of the ganglion (or section of the sympathetic), the constriction of the blood-vessels of the ear lasts a long time ; and we might justly assume that after their release from the influence of the sympathetic nerve, the blood-vessels of the iris under the influence of adrenalin also remain contracted for a long time.

[1] LEWANDOWSKY: *Loc. cit.*

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The facts that the palpebral fissure and nictitant membrane also show a paradoxical behavior, which was formerly adduced as a proof against the blood-vessel theory of the dilatation of the pupil, cannot be used in our case, as the muscles concerned in these phenomena might also be asphyxiated and thus cause the tonic contraction.

There is, however, one fact in our experience which seems to speak against this assumption. For the blood-vessels of the ear we have established that the simple section of the sympathetic is sufficient to prolong the constricting effect of adrenalin, and that the removal of the ganglion does not increase this effect. We are justified in assuming that the same holds good also for the other blood-vessels under the control of the sympathetic, including the vessels of the iris. Nevertheless administration of adrenalin causes dilatation of the pupil only after removal of the ganglion and not after section of the sympathetic nerve. Furthermore, we have often observed a dilatation of the pupil after injection of such a small dose of adrenalin as had no effect upon the blood-vessels of the ear. Finally the dilated pupil after injection of adrenalin persisted for hours after the death of the animal, when there could hardly have been any difference in the state of asphyxia between the tissues of the two eyes. Moreover, we ought also to point out that there is no gain in the assumption that the contraction of the smooth muscle fibres of the eye is due to asphyxia, and not to direct stimulation by the adrenalin. Since we know that this substance causes prompt contraction of the muscular sheath of the blood-vessels, why can we not assume that it can also cause the contraction of the dilator muscle of the pupil?

In presenting now our view of the nature of the paradoxical pupil-dilatation following the administration of adrenalin, we shall state at the outset that we see in this phenomenon an indication indeed, as we have stated at the beginning of our paper, that the ganglion sends normally impulses which prevent the dilatation of the pupil, — impulses which are opposite to those carried by the sympathetic nerve and which are represented, in part at least, by inhibition of the excitability of the dilator muscle. This agrees in general with the views of Kowalewsky and of Lewandowsky. We further agree with Lewandowsky and Anderson that the paradoxical phenomena are due, in part at least, to an increased excitability of the contractile tissues which have been freed from the influence of the ganglion. We finally also agree with the views of Lewandowsky and Langley that the suprarenal extract causes dilatation of the pupil and the

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other smooth muscles of the eye by directly stimulating the contractile tissues. In order to state our own view more explicitly, however, we have to go a little further.

It is now well established that the dilatation of the pupil is accomplished by the contraction of a muscle, the dilatator pupillæ, just as the constriction of the pupil is accomplished by the contraction of the constrictor pupillæ. Both muscles are exact antagonists, just as are such muscles as the extensors and flexors of an extremity; or as the rectus externus and internus of an eye. The constriction and dilatation of the pupil change with great rapidity, and the rapid play goes on nearly continually. Could such a rapid play ever take place if during the contraction of one muscle its antagonist should still be contracted and would simply have to be overpowered by sheer force? This seems hardly to require any discussion. Nobody claims it. The one antagonist is relaxed in proportion as the other contracts. What causes the relaxation? Is it simply a withdrawal of the impulse for contraction? We deal here with smooth muscle fibres whose contraction when once started would last a good many seconds, while the changes in the width of the pupil take place within a fraction of a second. The conclusion is unavoidable that the relaxation is an active one, — an inhibition of the contraction, not only of a tonus, as is often stated, but of an active contraction. And the further conclusion is, therefore, unavoidable that with each contraction of the dilator a simultaneous proportionate inhibition of the constrictor takes place, and, conversely, with each contraction of the constrictor a simultaneous inhibition of the dilator takes place.[1]

[1] I wish to append here the following remark: In my paper Das Schluckcentrum, seine Irradiationen, etc. (DU BOIS REYMOND'S Archiv für Physiologie, 1883, p. 209), I stated that in order to execute proper locomotion the nerve which controls flexion ought also to carry inhibitory fibres for the extension of an extremity. I have then called attention to the fact that such a proper antagonistic arrangement is to be found in the nervous mechanism of the respiratory function; for instance, the stimulation of the superior laryngeal, the second branch of the trigeminus and the central end of the splanchnic nerves, causes a contraction of the expiratory, and, simultaneously, an inhibition of the inspiratory muscles. I have also pointed out that from the "expiratory centres" (CHRISTIANI) active expiration and inhibition of inspiration can be produced; *i. e.* "that the centre for inhibition of inspiration is anatomically associated with that for active expiration and not with that for active inspiration." It was not until a good many years later that SHERRINGTON showed that stimulation of the central end of the nerve for the flexors (hamstring muscles) causes an inhibition of the extensor cruris; and that SHERRINGTON and H. E. HERING demonstrated that the same cortical area

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In this connection the experiments of E. Waymouth Reid[1] on the electrical currents of the iris during dilatation and constriction are of considerable interest. We know now that during the contraction of a muscle the electrical current is of a negative, and during its inhibition of a positive variation (Gaskell). Now Reid connected one pair of electrodes with the constrictor, and another pair with the dilator of the pupil, and observed the character of the currents which appeared during constriction and dilatation. When dilatation of the pupil was caused by stimulating the peripheral end of the sympathetic nerve, it was found that the current of the dilator showed a negative variation, while that of the constrictor showed a positive variation. This shows that the impulses sent through the sympathetic to the pupil are of two kinds: excitation to the dilator and inhibition to the sphincter. The reverse occurred when a constriction was caused by stimulating intracranially the oculo-motor nerve.

We have said above that the impulses sent by the ganglion are just the opposite to those carried by the sympathetic nerve. We can now say more precisely that the impulses of the ganglion are: inhibition of the dilator and excitation of the constrictor. As is usual with all antagonistic nerve fibres when they are stimulated simultaneously with the same strength, a resultant follows showing considerable predominance of the effects of one of the nerves. (Vagus

from which one group of muscles can be made to contract contains also the centre for inhibition of the antagonistic group of muscles. V. BASCH and EHRMAN, and FELLNER and v. ZEISSL have stated that the nerve trunk which contains the fibres inhibiting the circular muscle fibres of the small or large intestine, or the constrictor vesicæ, carries also the nerve fibres which cause contraction of the (antagonistic) longitudinal muscle fibres of the intestine or the detrusor vesicæ, and *vice versa*. They called this nervous arrangement of the organs "crossed innervation." SHERRINGTON designated the correlated phenomena which he observed on skeletal muscles "reciprocal innervation." In my second paper on inhibition (The Rôle of Inhibition, etc., Medical Record, 1902, p. 881), in which I have shown that the inhibition of one group during the activity of the antagonistic group of muscles, smooth or striated, is a wide-spread phenomenon through many of the mechanisms of animal life, I have termed this correlation: The Law of Crossed Innervation. In a recent paper, however (Archiv für Verdauungskrankheiten, 1903, ix, p. 450), in which I have dealt extensively with the same subject, I have given up, for reasons stated there, the term Crossed Innervation and designated the relations under discussion as The Law of Contrary Innervation. The inhibition of the sphincter pupillæ during an excitation of the dilator, or the inhibition of the dilator during a contraction of the pupil, is a good illustration of the Law of Contrary Innervation. S. J. M.

[1] E. WAYMOUTH REID: Journal of physiology, 1895, xvii, p. 438.

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and augmentor, sympathetic nerve and chorda, erigentes and hypogastrici, etc.) Of the cells of the ganglia some serve for the transmission of impulses from the central nervous system, brought to the ganglia through the sympathetic nerve fibres, and some generate new impulses. When the ganglion is stimulated, the effect upon those elements which carry the impulses from the central nervous system predominate. Hence stimulation of the ganglion will always cause a more or less distinct dilatation of the pupil (H. K. Anderson). It is possible also that the impulses sent by the ganglion are normally weaker than those sent by the central nervous system. When, however, by cutting the sympathetic nerves, the impulses carried by these fibres become eliminated, then the impulses of the ganglia come to the front. The constriction of the pupil after section of the sympathetic nerve is, perhaps, due not only to the elimination of the tonic impulses from the central nervous system, but also to the new activity of the previously overpowered antagonistic impulses of the ganglion. When, after a previous section of the sympathetic nerve, the ganglion is removed by excision (or evulsion), the very act of the excision [1] will, in the first place, act as an additional stimulus upon the postganglionic fibres which normally carry the subdued ganglionic impulses. The first effect of the removal will, therefore, sometimes be an increase in the constriction of the corresponding pupil; at all events it will not diminish the constriction. Since after removal of the ganglion there are no opposing impulses to overcome the stimulating effect of the section, or evulsion, this effect will wear off only slowly, after hours or even days. Gradually, however, the stimulating effect of the cut or tear disappears, and the pupil is now released from the hold of the ganglion. The first visible effect is a slight dilatation of the pupil again. But among other usually not noticeable effects is the increased excitability of the dilator muscle (after being freed from the inhibitory impulses of the ganglion), and the diminished readiness of the constrictor (after being deprived of the continually exciting impulses).

We now know that the suprarenal extract can cause a constriction as well as an inhibition of smooth muscle fibres. It causes constriction of the blood-vessels, contraction of the erector muscles of the hair, of the uterus of the rabbit, etc., and it causes inhibition of the

[1] Any cut can be felt for many hours and days; at the cut end of a nerve a stimulation is apparently going on for some time, even though its objective effect cannot be demonstrated in all cases.

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stomach, intestines, bladder, etc. Our assumption with regard to the pupil is that adrenalin affects it by exciting the dilator and inhibiting the constrictor. When the normal impulses of the superior cervical ganglia are present and active, they antagonize these effects. Subcutaneous injections or instillations which carry slowly and only minute doses of the suprarenal extract to the muscles of the iris, are easily overcome by the antagonistic impulses emanating from the ganglion, and even when injected intravenously the effect is very brief, since the effective dose is soon diminished and its balance is overpowered by the impulses from the ganglion. When, however, the ganglion and its antagonistic impulses are removed, and the stimulating effect of the cutting has vanished, or has been greatly diminished, the suprarenal extract meets with no antagonism, and hence the prolonged effect of an intravenous injection of adrenalin upon the blood-vessels and pupil, and the pupil-dilating effect of a subcutaneous injection or an instillation of even a comparatively small dose of it.

Furthermore, we are of the opinion that the long-lasting effect in these cases is due also to the absence of an antagonistic factor to cut it short, and it seems to us not unreasonable to assume that the continuation of the effect for some time after death is due also to the absence of a factor to remove it.

To sum up our view in a few words: we believe that the normal effect of the superior cervical ganglion is to inhibit the dilator and to stimulate the constrictor, and the effect of suprarenal extract is just the reverse of it, *i. e.* to excite the dilator and inhibit the constrictor. The extract can, therefore, show its proper effect only after the antagonistic activity of the ganglion is removed.

The "paradoxical" opening of the lids after subcutaneous injection of adrenalin can be explained on the same lines as that of the paradoxical pupil-dilatation; but we shall not enter here into a detailed discussion of it.

Neither shall we enter at present into a discussion of the obvious applicability of our interpretation also to the paradoxical pupil-dilatation caused by other factors (anæsthesia, etc.). We offered our interpretation of the phenomena observed by us as a working hypothesis. Its general applicability can wait until it is tested by more work.

The phenomena in the eye we have found while testing the hypothesis which we offered in explanation of the observations we made

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on the ear-vessels. We found that on intravenous injection of adrenalin the blood-vessels of the ear on the side on which the sympathetic and cervical nerves were cut remained constricted incomparably longer than on the normal side. We explained this by the assumption that the section of the nerves excluded the central vasodilating effect, assuming further that the suprarenal extract within the blood is soon reduced to the value of a weak stimulus which favors dilatation. In pursuance of this hypothesis, we found further that subcutaneous injection of moderate doses of adrenalin can indeed cause a dilatation of the ear-vessels of a normal animal, but that the same dose will invariably cause a long-lasting constriction of the ear-vessels when the sympathetic and cervical nerves had been previously cut. For the pupil we found that a subcutaneous injection has no effect on the normal, nor is the effect improved when the sympathetic nerves are cut. But subcutaneous injections, as well as instillations, have a marvellous effect when the superior cervical ganglion is removed. In other words, subcutaneous injection has practically no effect upon either vasoconstrictors or pupil; intravenous injection affects the pupil for a fraction of a minute, and the vasoconstrictors for four or five minutes. But after exclusion of the central influences, the constriction (of ear-vessels) lasts an hour and longer, and after removal of the ganglion, the dilatation of the pupil lasts for hours, whether the adrenalin was administered intravenously or subcutaneously. It is the central nervous system which interferes with the vasoconstriction, and it is the ganglion which interferes with the pupil-dilatation. Our first hypothesis reads that the central nervous system interferes with the vasoconstriction by means of the usual antagonism of the latter, *i. e.* by means of vasodilatation; our present hypothesis reads that the ganglion interferes with the pupil-dilatation by the usual antagonism to the latter, *i. e.* by means of the inhibition of the dilator and excitation of the constrictor.

We should point out the fact that the interference of the central nervous system concerns an organ which has only one set of muscle fibres — the constricting muscle fibres of the blood-vessels; while the interference of the ganglion concerns organs which have antagonistic sets of muscle fibres — the constrictor and dilator of the pupil (and also the muscles increasing and decreasing the width of the palpebral fissure). In our experience the interference with the nictitant membrane came from the central nervous system, — the membrane readily

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retracted on injection of adrenalin after section of the sympathetic nerve. According to the emphatic statement of Lewandowsky, there is no antagonistic muscle to the membrane. We should mention here that in our studies on the effect of the cervical sympathetic system on inflammation produced in the ear, we found that the course is much more unfavorably affected by the section of the sympathetic nerve than by the excision of the superior cervical ganglion. We had to explain these surprising conditions also by the assumption that the metabolic influences of the ganglion are exactly the reverse of those exerted by the sympathetic nerves.

We should once more call attention to the fact that our experiments with the subcutaneous injections of adrenalin throw sufficient light also upon another question of general character, namely, whether the suprarenal extract is destroyed through oxidation by the blood and tissues. The short duration of the rise of blood-pressure after an intravenous injection of the extract was explained by Szymonowicz[1] and Cybulski and especially by Langlois[2] and his co-workers[3] to be due to a rapid oxidation of the extract by the blood. The failure to obtain the effect upon the pupils as well as upon the blood-pressure by subcutaneous injections was also explained through the oxidation of the extract by the tissue (Lewandowsky, Boruttau). Very recently Embden and v. Fürth[4] have in a number of experiments mixed the extract (suprarenin, v. Fürth) with blood and various tissues and found that the effect of the extract did not suffer even by prolonged contact. Against this evidence it could, however, be claimed that living tissues might act differently upon the extract than the dead substances which were used by these authors.

Our many experiments which have shown that the effect upon the blood-vessels as well as upon the pupil can be considerably prolonged if only the sympathetic nerves were cut or the superior cervical ganglion removed, demonstrate sufficiently that the extreme brevity of the effect, or the failure to produce any effect, cannot be due to a rapid destruction of the extract through oxidation by the tissues. However a most conclusive demonstration how little the living tissues affect the activity of the extract is to be found in our

[1] SZYMONOWICZ: Archiv für die gesammte Physiologie, 1896, lxiv, p. 143.

[2] LANGLOIS: Comptes rendus de la société de biologie, 1897, xlix, p. 524.

[3] ATHANASIUS and LANGLOIS: *Ibid.*, p. 575.

[4] EMBDEN and v. FÜRTH: Hofmeister's Beiträge für chemische Physiologie und Pathologie, 1903, iv, p. 421.

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experiments with subcutaneous and intramuscular injections of adrenalin into an extremity beneath a ligature. Here the adrenalin remained in intimate contact with the tissues for two hours and longer without losing any of its effects. In fact, the effects set in more promptly and rapidly than after a simple subcutaneous injection.

These experiments seem to speak also against the supposition made by Embden and v. Fürth that the suprarenal extract might be partly destroyed by the alkalinity of the blood. We are not aware that the alkalinity of the tissues is markedly less than that of the blood. Nevertheless, the efficiency of the extract, as our experiments show, did not seem to suffer by a prolonged intimate contact with the living tissues.

Our experiments teach also, we believe, an important pharmacodynamic lesson. The suprarenal extract or adrenalin is now a much-employed drug. The general knowledge of its activity was derived from studies made upon normal animals. From these studies it was learned that subcutaneous injections have hardly any influence upon blood-pressure and have no influence upon the pupil. We have shown that when the sympathetic is cut or the superior cervical ganglion is removed, such injections of adrenalin exert a very prompt influence indeed and one of much longer duration than in normal animals. Now our knowledge of the effects of all drugs and animal extracts is derived from studies upon normal animals. Is that sufficient? What is their effect upon animals which are deprived of certain nervous or other influences? Might we not find in many, if not in most, of the cases, influences of an entirely different character? This is the more important, since we are interested in drugs chiefly on account of their possible influence upon the animal organism when the latter is in a pathological condition.

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Reprinted from the American Journal of Physiology.
VOL. IX. — MARCH 2, 1903. — NO. I.

AN EXPERIMENTAL STUDY OF THE SUGAR CONTENT AND EXTRAVASCULAR COAGULATION OF THE BLOOD AFTER ADMINISTRATION OF ADRENALIN.

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[*Carried out under the auspices of the Rockefeller Institute for Medical Research at the Laboratory of Physiological Chemistry, of Columbia University, at the College of Physicians and Surgeons, New York.*]

INTRODUCTION.

EARLY in 1902 the discovery was made by Herter and Richards[2] that the injection of solutions of adrenalin chloride into the peritoneal cavity of dogs was followed by an intense though transient glycosuria. It was also found that the application of adrenalin solution directly to the surface of the pancreas produced a similar effect. As a result of a number of experiments in this direction, the suggestion was offered that this form of glycosuria was in reality of pancreatic origin.

In extending these observations, Herter and Wakeman[3] have found that the power of adrenalin to produce glycosuria, when applied to the pancreas, is not specific but is shared with various substances. The number of such substances is comparatively large, and apparently the only quality common to the series is a reducing activity. A seeming exception to this rule was found in potassium cyanide. When solutions of this substance were applied to the pancreas in amounts far too small to produce general toxic symptoms, glycosuria resulted. This substance, like hydrocyanic acid, while it has no reducing power, exerts a specific action on the animal cells in preventing them from absorbing oxygen.[4] It is natural to suppose that, in the absence of the normal amount of oxygen in the cell, an excess

[1] Research scholar of the Rockefeller Institute.

[2] HERTER and RICHARDS: The medical news, 1902, lxxx, p. 201.

[3] HERTER and WAKEMAN: Virchow's Archiv für pathologische Anatomie und Physiologie und für klinische Medicin, 1902, clxix, p. 479; HERTER: The medical news, 1902, lxxx, p. 867.

[4] GEPPERT: Zeitschrift für klinische Medicin, 1889, xv, p. 208; *Ibid.*, p. 307.

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of reducing substances may be formed. It is possible that these substances may act in a manner comparable to those of the above-mentioned series in bringing about the excretion of sugar. From the facts brought out by their observations in this regard, Herter and Wakeman are inclined to attribute the production of glycosuria upon the application of adrenalin and other substances to the pancreas to a toxic action on the cells of that gland which is closely connected with the power of reduction.

If this view of the matter is correct, an important relationship suggests itself between this form of experimental glycosuria and conditions in the human organism which may give rise to an excretion of sugar. The fact that many organs of the body may form reducing substances capable of easy oxidation which may reach the pancreas in the blood stream, carries with it the possibility that, if the normal balance between the amount of these substances and the oxidizing power of the pancreas be disturbed, the production of glycosuria may occur.

Concerning the mechanism through which adrenalin brings about the excretion of sugar no positive statements can as yet be made. The work of Minkowski[1] and his followers, which has furnished the basis of the belief in the existence of an internal secretion of the pancreas exercising a controlling influence on carbohydrate metabolism, justifies an assumption that the sugar elimination is the result of an alteration in the nature, activity, or amount of this secretion. The glycosuria-producing effect of injury of certain parts of the central nervous system,[2] and the increase in sugar formation in the liver which follows stimulation of the cœliac plexus[3] or of the vagus nerve,[4] may lead to the supposition that adrenalin glycosuria results from the action of a nervous mechanism. Finally, it is known that under the abnormal conditions which follow the injection of phlorhizin[5] or chromic acid,[6] glycosuria may occur, owing to an increase in the permeability of the kidney cells. The possibility that adrenalin

[1] MINKOWSKI: Untersuchungen über den Diabetes Mellitus nach Extirpation des Pancreas, Leipzig, 1893.

[2] CL. BERNARD: Leçons sur la physiologie et la pathologie du system nerveux, Paris, 1858, i, p. 401.

[3] A. and E. CAVAZZANI: Centralblatt für Physiologie, 1894, viii, p. 33.

[4] LEVENE: Centralblatt für Physiologie, 1894, viii, p. 337.

[5] v. MERING: Zeitschrift für klinische Medicin, 1889, xvi, p. 431.

[6] KOSSA: Archiv für die gesammte Physiologie, 1901–1902, lxxxviii, p. 627.

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glycosuria is the immediate result of changes in the kidney has not yet been excluded.

Whatever may be the manner by which the effects of adrenalin are brought about, it is probable that the mechanism involved is one which is active, though in a different degree, under normal conditions. A determination of the identity of the mechanism is therefore of importance, not only in explaining the phenomenon in question, but also from the fact that it may throw light on some of the processes connected with the normal metabolism of carbohydrate within the organism.

Before such a determination can be made, however, a more accurate knowledge of the internal conditions antecedent to the excretion of sugar is necessary. With this end in view we have made a somewhat detailed study of the sugar in the blood, after intraperitoneal injection of adrenalin, as well as after application of that substance to the pancreas.

Sugar Content and Coagulation of Arterial Blood after Treatment with Adrenalin.

It has long been known that the glycosuria produced by extirpation of the pancreas,[1] puncture of the floor of the fourth ventricle,[2] and poisoning with certain substances, such as carbon monoxide,[3] is the immediate result of an increased accumulation of sugar in the blood. On the other hand, injections of phlorhizin[4] are followed by the excretion of sugar due to the effect on the kidney. In the latter case the percentage of sugar in the blood never rises above normal, and may even fall below that amount. To determine in which class adrenalin glycosuria belongs, we have made a number of determinations of the sugar content of the blood of dogs which had been subjected to treatment with adrenalin. In this series also we have attempted to ascertain the rapidity with which this substance acts, and the course and duration of its influence.

Method of collection and analysis of blood. — Healthy, well-nourished dogs were anæsthetized with pure ether, a cannula introduced into a femoral artery, and a portion of blood taken. The solution of adrena-

[1] Minkowski: *Loc. cit.*

[2] Cl. Bernard: *Loc. cit.*

[3] Senff: Ueber den Diabetes nach der Kohlenoxydathmung, Inaugural dissertion, Dorpat, 1869.

[4] v. Mering: *Loc. cit.*

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lin chloride[1] was then injected by a hypodermic syringe into the peritoneal cavity or, after an incision through the abdominal wall, was painted on the surface of the pancreas with a soft brush. Portions of blood were then drawn from the femoral artery at various intervals.

Having in mind the possible production of glycosuria by means of anæsthetics,[2] as well as by asphyxia,[3] care was taken to keep the anæsthesia as light and as constant as possible. Moreover, we believe that this factor may be left out of account in these experiments, since the control portion of blood, taken before adrenalin treatment, was collected under the same conditions of anæsthesia as the subsequent portions which are compared with it.

The portions of blood were analyzed according to the following procedure. The blood was drawn directly into a beaker containing a solution of phosphotungstic acid in dilute hydrochloric acid.[4] The beaker was counterpoised on a balance and the blood weighed immediately after its withdrawal from the artery. On boiling this mixture the blood proteids are precipitated in a granular form leaving a water-clear fluid free from proteid. The precipitate was washed thoroughly with hot water, a process which is rendered easy by its porosity and its friable character. The combined filtrate and washings were nearly neutralized with sodium hydroxide and evaporated to small bulk on the water bath. The evaporated residue was made up to known volume (50–100 c.c.) with water, and was filtered. The reducing power of this solution was determined by the Allihn method. The results were calculated in terms of dextrose from the weight of the metallic copper. The figures given represent the averages of closely agreeing duplicates.

Method of determining coagulation. — In one of our early experiments we noticed that a portion of blood drawn for the purpose of rinsing the cannula clotted very rapidly. As a result of this observation, in a number of later experiments we have taken separate por-

[1] In all the experiments outlined in this paper, the adrenalin chloride solution ($\frac{1}{1000}$) prepared by Parke, Davis, & Co., by the method of Takamine, was used.

[2] CUSHNY: Pharmacology and Therapeutics or the action of drugs, 1899, p. 160.

[3] DASTRE: Comptes rendus des séances de l'academie des sciences, 1879, lxxxix, p. 669.

[4] This solution contained 70 gms. of phosphotungstic acid and 20 c.c. of hydrochloric acid, sp. gr. 1.20, in a litre. About 5 c.c. are sufficient to completely precipitate the proteids in 1 gm. of blood.

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tions of blood to be tested regarding this point. The amount drawn in each case was 2 c.c., collected in a graduated cylinder of 5 c.c. capacity. The time which elapsed between the collection of the blood and the time at which the cylinder could be inverted without loss of its contents, was noted as the time of the coagulation of the blood.

The results of our determinations are given in Table I, pages 40, 41.

These experiments show unmistakably that the administration of adrenalin chloride either by intraperitoneal injection or by painting it upon the pancreas is followed by a marked increase of sugar in the blood. The increase is very noticeable within the first five minutes after the application and reaches its maximum within three hours. A very gradual fall then ensues, which may continue until the percentage of sugar becomes subnormal (Exp. 2). In a dog recently fed (Exp. 1), the blood sugar may be double the normal quantity fourteen hours after the injection. A marked rise occurred in the case of a dog (Exp. 7) which had been starved for six days. In Experiment 8 a fatal dose of adrenalin was given. A slight increase in the sugar content of the blood occurred shortly after. One minute before death ensued, twenty-four hours after injection, the percentage of sugar was approximately normal.

Simultaneously with the production of hyperglycæmia, an effect on the coagulability of the blood is observed. In every case, without exception, the time of coagulation is lessened after adrenalin is given. This diminution is equal in some cases to four-fifths of the coagulation time of the control.

Arthus[1] has shown that the time of coagulation decreases if the blood is allowed to come in contact with blood already clotted or with an exposed tissue surface. Special care has been taken therefore in these experiments to remove the clot from the cannula before each collection. Furthermore the portion for the coagulation test was collected just after that for sugar analysis, a circumstance which insures the rinsing of the cannula.

The recent observation[2] by the same author that the mere withdrawal of large amounts of blood from the body hastens the coagulation of subsequent portions, raises the question whether the results which we have observed may have been due to loss of blood alone. To test this point, a control experiment was made in which the

[1] ARTHUS: Journal de physiologie et de la pathologie générale, 1902, iv, p. 283.
[2] ARTHUS: *Ibid.*, p. 273.

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TABLE I.

SUGAR CONTENT AND COAGULATION OF ARTERIAL BLOOD AFTER TREATMENT WITH ADRENALIN.

No. of experiment.	Weight of dog. Kilo.	Time since last fed.	Amount of blood withdrawn. Grams.	Time when taken.	Sugar. Per cent.	Time of coagulation.	Remarks.
1	8.5	12 hrs.	29.0	9.30 P. M.: normal	0.16		Etherized at 9.15 P. M.; 8 c.c. adrenalin chloride solution ($\frac{1}{1000}$) injected intraperitoneally at 9.35 P. M.; anæsthesia continued till 12.30 A. M.; again etherized on following morn. at 11.30.
			30.0	10.35 P. M.: 1 hr. after injection	0.42		
			25.0	11.30 P. M.: 2 hrs. " "	0.56		
			24.0	12.30 A. M.: 3 " " "	0.52		
			33.75	11.45 A. M.: 14 " " "	0.37		
						min. sec.	
2	7.6	32 hrs.	29 0	5.44 P. M.: normal	0.258	4 00	Etherized at 5 30 P. M.; 8 c.c. of adrenalin chloride solution ($\frac{1}{1000}$) injected intraperitoneally at 5.47 P. M.; anæsthesia continued till 7.00 P. M.; again etherized at 10.30 P. M., also at 10.30 A. M. next morning.
			15.0	5.52 P. M.: 5 min. after injection	0.357	2 15	
			11.5	5.57 P. M.: 10 " " "	0.400	2 00	
			10.7	6.20 P. M.: 33 " " "	0.437	2 20	
			11.8	6.47 P. M.: 1 hr. " "	0.430	4 10	
			10.8	10.35 P. M.: 5 hrs. " "	0.307	3 05	
			27.3	10.38 A. M.: 17 " " "	0.099	3 00	
3	12.0	48 hrs.	22.4	3.00 P. M.: normal	0.112		Etherization continued throughout experiment; 3 c.c. of adrenalin chloride solution ($\frac{1}{1000}$) applied to pancreas with a brush at 3.08 P. M.
			21.5	3.15 P. M.: 7 min. after painting	0.182		
			22.5	3.23 P. M.: 15 " " "	0.178		
			19.7	3.38 P. M.: 30 " " "	0.188		
			20.0	4.08 P. M.: 1 hr. " "	0.204		
			21.4	5.06 P. M.: 2 hrs. " "	0.214		
			20.8	6.00 P. M.: 3 " " "	0.165		
4	7.1	48 hrs.	21.9	7.55 P. M.: normal	0.173		Etherized from 7.45 till end of experiment; 4 c.c. adrenalin chloride solution ($\frac{1}{1000}$) painted on surface of pancreas at 8.12 P. M.
			27.3	8.17 P. M.: 5 min. after painting	....		
			21.1	8.27 P. M.: 15 " " "	0.277		
			20.4	8.42 P. M.: 30 " " "	0.237		
			21.8	9.12 P. M.: 1 hr. after painting	0.291		
			21.3	10.12 P. M.: 2 hrs. " "	0.256		

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						min.	sec.	
5	13 2	24 hrs.	19.9	10.45 A.M.: normal	0.239	5	20	Ether given throughout the experiment; 3 c.c. of adrenalin chloride ($\frac{1}{1000}$) applied to pancreas at 10.55 A.M.
			23 8	11.00 A.M.: 5 min. after painting	0.291	3	00	
			25 8	11.10 A.M.: 15 " " "	0.354	1	57	
			19.4	11.25 A.M.: 30 " " "	0 388	2	07	
			20.3	11.54 A.M.: 1 hr. " "	0.433	1	12	
			21.2	12.54 P.M.: 2 hrs. " "	0.477	1	35	
			19.3	1.54 P.M.: 3 " " "	0.519	1	15	
			20.0	2.55 P.M.: 4 " " "	0.465	0	50	
6	7.0	24 hrs.	17.9	4.52 P.M.: normal	0.131	6	50	Ether given throughout experiment; 2 c.c. of adrenalin chloride solution ($\frac{1}{1000}$) applied to pancreas at 5.00 P.M.
			17.0	5:05 P.M.: 5 min. after painting	0.205	3	05	
			20.5	5.15 P.M.: 15 " " "	0.217	1	40	
			19.0	5.30 P.M.: 30 " " "	0.264	1	20	
			18 3	6.00 P.M.: 1 hr. " "	0.315	1	47	
			17.0	7.00 P.M.: 2 hrs. " "	0.371	1	40	
7	7.4	6 days	17.0	3.34 P.M.: normal	0.154	2	00	Ether given throughout experiment; 3 c.c. of adrenalin solution applied to pancreas at 3.43 P.M.
			17.6	3.49 P.M.: 6 min. after painting	0.192	2	25	
			18.2	4.01 P.M.: 18 " " "	0.173	2	40	
			17.0	4.22 P.M.: 29 " " "	0.191	2	05	
			16.5	4.58 P.M.: 1 hr. 15 min. aft. painting	0.206	2	15	
			17.7	5.43 P.M.: 2 hrs. after painting	0.143	1	55	
			18.5	6.37 P.M.: 3 " " "	0.169	1	16	
8	13.7	5 days	14.9	9.23 P.M.: normal	0.138	4	30	Anæsthetized with mixt. of chloroform and ether; 12 c.c. of adrenalin chloride sol. ($\frac{1}{1000}$) injected intraperitoneally at 9.33.30 P.M.; kept under ether till 11.15 P.M.; etherized at 10.30 following morning; animal very much prostrated; no ether given at night; characteristic bloody mucous passed in fæces; died 1 min. after last portion of blood was collected.
			15.6	9.35 P.M.: 1 min. 30 sec. aft. injection	0.104	1	20	
			11.3	10.18 P.M.: 45 min. after injection	0.095	2	20	
			10.4	11.03 P.M.: 1 hr. 30 min. aft. injection	0.199	3	15	
			19.0	10.31 A.M.: 13 hrs. after injection	0.058	2	40	
			26.5	9.38 P.M.: 24 " " "	0.102	5	30	

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amount of blood taken was very small. The details of the experiment are as follows:

A small dog, 5.3 kilos in weight, which had received no food for twenty-eight hours previous to the experiment, was etherized and a cannula introduced into the femoral artery. 2 c.c. of blood collected at 2.34 P. M., coagulated in 5 min. 50 sec. An incision was made through the abdominal wall and 2 c.c. of adrenalin solution applied to the pancreas with a soft brush at 2.43 P. M. Subsequent samples of blood (2 c.c. each) coagulated as follows:

5 min.	after	adrenalin	application	(2.48 P. M.)	coagulated	in	1 min.	45	secs.
16 "	"	"	"	(2 59 P. M.)	"		2 "	0	"
33 "	"	"	"	(3.16 P. M.)	"		2 "	32	"
1 hr.	"	"	"	(3.45 P. M.)	"		3 "	10	"

Other control experiments in which large amounts of blood were removed, and no treatment with adrenalin given, show no such marked changes as are seen in the observations in Table I.

THE SOURCE OF THE EXCESS OF SUGAR IN THE BLOOD IN ADRENALIN GLYCOSURIA, AS INDICATED BY COMPARATIVE ANALYSIS OF BLOOD COLLECTED SIMULTANEOUSLY FROM THE PORTAL AND HEPATIC VEINS AND THE FEMORAL ARTERY.

The results just detailed show clearly that the phenomenon of adrenalin glycosuria is due to an increase of sugar in the blood. The source of this excess of sugar is of great importance in determining the mechanism by which this effect is brought about. We have endeavored to trace the source of the sugar by means of analysis of blood taken simultaneously from the portal and hepatic veins and from the femoral artery. In these experiments it was necessary to collect successive portions of blood at various intervals from the same blood-vessel without interfering with the normal circulation in that vessel. The methods which have hitherto been devised for the collection of portal and hepatic blood appeared to be inadequate for our purpose, as well as somewhat difficult and uncertain of application.[1] We have therefore made use of an original method, the description of which follows. A cannula of special design[2] (see accompanying diagram) is the essential feature of the method.

[1] For description of older methods, see SEEGEN: Die Zuckerbildung im Thierkörper, 2 Auflage, Berlin, 1900, p. 66.

[2] The special cannulas used in these experiments were made for us very skilfully by Mr. John T. Hoyt of the Department of Physiology in this institution.

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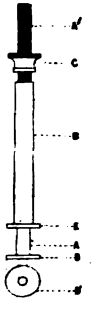
General method. — The method of fixing the cannula into a vein is as follows: The vessel is carefully exposed, and the outer connective tissue sheath dissected away. Loose ligatures are passed about the vessel at each end of the cleared portion, which should be about 2 cm. in length. Before introducing the cannula, its parts should be so arranged that the flange of the outer tube is separated by a space of about 0.5 cm. from that of the inner tube. The brass rod should be in position, totally filling up the bore of the inner tube. The ligatures about the vessel are then tightened momentarily by an assistant, and a longitudinal slit made in the wall of the vessel between them. This slit should be a trifle shorter than the diameter of the flange. The flange of the inner tube is then introduced into the interior of the vessel through the slit, the outer tube pressed down till the wall of the vessel is held tightly between the two flanges, and the nut screwed down so that the hold is retained. The ligatures are then loosened and the normal circulation is resumed. With a little practice the operation can be accomplished with no loss of blood and an interruption of the circulation of only thirty to forty seconds. On connecting a rubber tube with the inner tube of the cannula and removing the brass rod, blood can be withdrawn at pleasure.



EXPLANATION OF DIAGRAM. — The cannula consists of three parts, viz.: an inner tube (*A*), an outer tube (*B*), and a nut (*C*). The inner tube is about 6.5 cm. in length. Its outer diameter is 4 mm., its inner diameter, 2 mm. On it a screw thread (*A'*) is cut, extending for 3.5 cm. from one end. The other end is provided with a flange (*D*) 9 mm. in diameter. The end view of this flange is shown at *D'*. The outer tube (*B*), 3.5 cm. long, fitting closely over the inner tube, terminates in a flange (*E*) similar to the one on the latter. By means of the nut the two tubes may be held in such a position that their flanges are in close contact. All parts of the instrument are made of brass. The cannula is also provided with a brass rod (not shown in the diagram) about 28 cm. long, of such a thickness that it fits closely into the bore of the inner tube. A small shoulder brazed on the rod at the proper point insures the complete occlusion of the cannula when desired.

In our experiments to determine the sugar content of the blood flowing to and from the liver, we have introduced cannulas of this type into the portal vein at its juncture with the pancreatico-duodenalis, and into one of the larger hepatic veins at a point between the liver and the diaphragm.

In order to expose the vessels, a transverse cut was made through the abdominal wall, following the curvature of the free border of the ribs and extending for about three inches on each side of a point on the median line just below the xyphoid cartilage. Bleeding was prevented by ligaturing the vessels which it was necessary to cut. The abdominal organs were protected from exposure by cloths moistened with warm saline solution. The time necessary for the operation and the introduction of the cannulas into both veins usually amounted to about one hour.

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In drawing blood from the hepatic vein it was necessary to use a suction pump to overcome the negative pressure, which is very manifest in the venous circulation at this point. For this purpose, the beaker containing the phosphotungstic mixture, previously weighed, was placed under a small bell-jar which was connected by its lower opening with a suction pump. A small glass tube inserted through the rubber stopper which closed the upper opening of the bell-jar, and terminating at a point directly over the beaker, served to conduct the blood into the precipitating fluid. On connecting the cannula in the hepatic vein with the glass tube, and applying suction, the estimated amount of blood is easily obtained.

For collecting arterial blood, a glass cannula of the ordinary type was introduced into the femoral artery.

The order of procedure in obtaining the portions of blood simultaneously was as follows: The beakers which were to hold the femoral and portal blood were counterpoised on balances placed at the side of the operating table. Small glass tubes were clamped in a position to lead the blood into them. The beaker for collection of hepatic blood was placed under the bell-jar arranged as described above. The brass rods were removed from the cannulas in the portal and hepatic veins, and connection with the proper glass tubes made, passage of blood being prevented by clamping the rubber tubes. The connection between the cannula in the femoral and the third glass tube was made. At a given signal the clamps were removed and the blood collected. The brass rods were immediately replaced in the cannulas, and the portions of blood weighed. The time necessary for collection of all three portions of blood has seldom amounted to more than ten or fifteen seconds.

The analysis of the blood was carried out in a manner exactly similar to that previously described.

By means of these methods we have succeeded in obtaining blood simultaneously from the three vessels mentioned, both before and after the application of adrenalin chloride to the surface of the pancreas. The results of sugar determination are given in the table on page 45.

The results of Experiments 1, 2, 3, and 6 present, we believe, a true picture of the events in this connection succeeding the application of adrenalin to the pancreas. The samples of blood taken before treatment with adrenalin agree fairly closely in their sugar content. On the ground of these figures it cannot be said that the amount of sugar in the blood issuing from the liver is greater than that of the femoral artery or portal vein. After treatment with adrenalin, however, the relations are changed. In Experiment 3, four minutes after

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TABLE II. — Sugar Content of Blood collected simultaneously from the Hepatic and Portal Veins and Femoral Artery before and after Treatment with Adrenalin.

No. of exp.	Weight. Kilo.	Time since last fed. Hrs.	Amount of blood from: Femoral artery. Gms.	Portal vein. Gms.	Hepatic vein. Gms.	Time of collection. P. M.	Time of application of adrenalin. P. M.	Time of collection compared with time of application.	Sugar in blood from: Femoral artery. Per cent.	Portal vein. Per ct.	Hepatic vein. Per ct.	Remarks.
1	9.0	72	17.5	31.4	31.2	9.31	9.37	6 min. before	0.10	0.10	0.08	Chest opened [1] and artificial respiration employed.
			14.4	11.4	30.0	9.57		20 min. after	0.15	0.08	0.25	
2	9.2	24	12.7	14.9	16.8	4.23	4.39	16 min. before	0.160	0.165	0.164	At time of first collection of blood, pancreas and intestines normal in appearance. At second collection, pancreas somewhat congested, intestines somewhat cyanotic. At third drawing of blood, the pancreas was very much congested and small intestine very blue.
			13.4	14.4	16.2	4.49		10 min. after	0.247	0.197	0.284	
			13.8	8.4	12.3	5.20		41 " "	0.322	0.245	0.346	
3	7.8	20	12.9	13.7	21.0	4.21	4.40	19 min. before	0.159	0.169	0.136	At time of first and second collections of blood, the pancreas and small intestines were normal in appearance. When the third and fourth series were taken, these organs were much congested.
			12.6	17.6	13.2	4.44		4 min. after	0.187	0.167	0.201	
			12.4	14.5	13.3	5.06		26 " "	0.215	0.187	0.252	
			13.4	13.6	19.1	5.46		66 " "	0.208	0.207	0.209	
4	15.65	24	15.6	15.5	13.85	5.29	5.42	13 min. before	0.311	0.282	0.316	At first drawing of blood, the pancreas and intestines were normal. At the second, the small intestine was congested and cyanotic.
			13.6	20.1	15.45	6.09		27 min. after	0.385	0.314	0.348	
5	5.35	26	15.3	16.5	13.8	3.15	3.25	10 min. before	0.221	0.227	0.210	Pancreas and intestines normal at time of first collections. Pancreas congested and small intestine congested and cyanotic at time of second.
			13.4	11.7	12.0	3.46		21 min. after	0.242	0.158	0.240	
6	13.2	25	14.7	15.15	11.5	5.26	5.36	10 min. before	0.201	0.181	0.208	Pancreas and intestines normal in appearance at first collections. Pancreas congested and intestines congested and cyanotic at time of second.
			16.0	12.4	19.1	6.01		25 min. after	0.208	0.223	0.227	

[1] In this experiment only was it found necessary to open the chest in order to insert the cannula into the hepatic vein.

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the application of the substance to the gland the sugar content of the arterial blood rises 0.028 per cent, that of the portal blood remains practically the same, while the increase in reducing power of the blood emerging from the liver amounts to 0.065 per cent. The same relation, though on a higher plane, is apparent twenty-six minutes later. Sixty-six minutes after, the sugar percentage from all the vessels is approximately the same. Precisely similar results are obtained in Experiments 1 and 2, and in a lesser degree in Experiment 6. Judging from the results of these analyses then, a formation of sugar in the liver must be the cause, in part at least, of the increase of sugar in the blood.

Experiments 4 and 5 apparently form exceptions to this conclusion. It will be noticed, however, that the percentage of sugar in the samples taken before adrenalin treatment are abnormally high, especially in Experiment 4. It is possible that the mechanism which takes part in the production of adrenalin glycæmia has already been affected by the operative disturbance. It is not an unfair assumption that the additional impulse given by the application of adrenalin is on that account less effective and its result more transient. Consequently at the time of the collection of the second portions of blood the secretion of sugar is lessening. The same reasoning may hold good with regard to Experiment 5.

Comparison of the sugar content of the portal blood with that from the femoral artery and hepatic vein in Experiments 1, 2, 3, and 5 shows that the increase of sugar following treatment of the pancreas with adrenalin is least in the portal vein. While in the control samples the sugar percentage of the portal is as high or higher than that of the femoral or hepatic blood, after treatment with adrenalin, it is lower in every case. In this connection we would call attention to certain changes in the appearance of the organs of the abdomen. At the time of collection of the first samples of blood the appearance of the intestines and pancreas was normal. As the experiment proceeded, however, the pancreas became congested and the intestines cyanotic. The latter symptom is due, probably, to a partial obstruction of the circulation by the formation of a clot at the flange of the cannula. The effect of this partial obstruction is a partial asphyxia of the tissues. That the *relative* decrease in the sugar of the portal blood is dependent upon increased utilization within the tissues through which it passes there can be no doubt. Whether this consists in a mere increased oxidation of sugar, owing to the increased

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TABLE III.

SUGAR CONTENT OF BLOOD TAKEN SIMULTANEOUSLY FROM THE HEPATIC AND PORTAL VEINS AND THE FEMORAL ARTERY, TREATMENT WITH ADRENALIN BEING OMITTED.

No. of experiment.	Weight. Kilo.	Time since fed. Hours.	Amount of blood from			Time of collection.	Sugar in blood from			Remarks.
			Femoral artery. Grams.	Portal vein. Grams.	Hepatic vein. Grams.		Femoral artery. Per cent.	Portal vein. Per cent.	Hepatic vein. Per cent.	
1	13.6	22	14.5	13.1	16.4	4.45 P. M.	0.236	0.213	0.210	Appearance of the pancreas and intestines normal throughout experiment.
			12.9	12.9	11.6	5.05 P. M.	0.220	0.193	0.242	
			13.6	11.7	13.7	5.30 P. M.	0.190	0.198	0.205	
			13.5	15.1	13.7	6.05 P. M.	0.217	0.185	0.217	
2	7.7	29	12.0	14.2	13.8	4.24 P. M.	0.174	0.160	0.166	Intestines became somewhat blue at the time of second and third collections of blood. Pancreas normal.
			14.3	13.5	16.7	4.46 P. M.	0.183	0.179	0.188	
			15.8	11.9	13.2	5.16 P. M.	0.194	0.173	0.174	
3	17.15	48	13.5	15.1	13.3	12.10 P. M.	0.215	0.174	0.216	Intestines somewhat blue at time of second blood collection. Pancreas normal.
			14.5	25.8	14.4	12.42 P. M.	0.264	0.218	0.260	
4	5.7	24	13.5	13.7	18.15	11.50 A. M.	0.188	0.176	0.194	Intestines somewhat blue at second collection. Pancreas normal.
			13.9	13.7	20.7	12.30 P. M.	0.182	0.198	0.190	
5	11.4	24	14.7	14.1	14.9	2.59 P. M.	0.206	0.204	0.202	Pancreas and intestines normal.
			13.4	12.7	17.0	3.34 P. M.	0.175	0.149	0.185	

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supply of that substance, or whether there is a decomposition of another character in increased amount, owing to lack of oxygen in the tissues, our experiments do not decide.

In referring to Experiments 4 and 5, the idea has been expressed that the high sugar content found in the control samples was possibly due to the effects of operative disturbance. The question might naturally be raised as to whether the effects noted in our other experiments might not be due to that cause rather than to the influence of adrenalin. To settle this point we have made a series of five control experiments in which the blood was collected in a manner similar to that described, the treatment with adrenalin being omitted. The results are given in Table III, page 47.

These figures show an essential difference from those given in Table II. In only one case does the blood of the hepatic vein contain considerably more sugar than that of the femoral artery. In only one experiment (3) is there an essential rise in the sugar of the femoral blood. The results indicate, therefore, that while in a small percentage of experiments carried out according to this method, the operation may give rise to a hyperglycæmia similar to that produced by adrenalin, in the majority of cases we are justified in attributing the results to the action of adrenalin.

Sugar in the Blood of the Pancreatico-duodenal Vein after Treatment of the Pancreas with Adrenalin.

It has been shown in the experiments of Series II, page 45, that in the hyperglycæmia which follows the application of adrenalin to the pancreas, the increase of sugar is least in the blood of the portal vein. We have attributed this circumstance to an increased decomposition of sugar in the intestinal tissues, and have suggested that it may be connected with a partial obstruction in the circulation of the blood through those tissues. To ascertain whether the congestion of the pancreas which is regularly observed after treatment of that gland with adrenalin takes part in this phenomenon, we have tested the blood from the pancreatico-duodenal vein. Though an increased decomposition of sugar in the pancreas would be at variance with our ideas regarding the events taking place there, its possibility has not been positively excluded.

The method of collecting blood was as follows: A cannula of the design previously described was introduced into the portal vein at a

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TABLE IV.

SUGAR CONTENT of BLOOD FROM THE PANCREATIC VEIN BEFORE AND AFTER APPLICATION OF ADRENALIN TO THE PANCREAS.

No. of experiment.	Wgt. Kilo.	Time since fed. Hours.	Amt. of blood from Femoral artery. Grams.	Amt. of blood from Pancreatic vein. Grams.	Time of collection. P. M.	Time of application of adrenalin. P. M.	Time of collection compared with time of application.	Sugar in blood from Femoral artery. Per cent.	Sugar in blood from Pancreatic vein. Per cent.	Appearance of pancreas.
1	13.4	22		9.5	4.15	4.27–4.28.05	13 min. before.		0.202	Normal.
				14.7	4.31.05		3 min. after.		0.275	Pale.
				10.4	4.37		8 min. 55 sec. after.		0.307	Very slightly congested.
2	10.4	24		12.4	3.57	4.04 30–4.06.05	9 min. before.		0.209	Normal.
				10.6	4.06.30		25 sec. after.		0.277	Very pale.
				10.1	4.11.30		5 min. 25 sec. after.		0.287	Somewhat congested.
3	18.6	30		12.8	4.02	4.08–4.09.20	7 min. 20 sec. bef.		0.180	Normal.
				13.2	4.10.05		45 sec. after.		0.212	Very pale.
				9.4	4.17.40		8 min. 20 sec. after.		0.215	Somewhat congested.
				15.1	4.25.35		16 min. 15 sec. after.		0.236	Very much congested.
			16.9		4.27		17 min. 40 sec. after.	0.226		
				16.9	4.35.35		26 min. 10 sec. after.		0.218	Very much congested.
			16.3		4.38		28 min. 40 sec. after.	0.210		

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point just opposite the entrance of the pancreatico-duodenalis. Loose ligatures were placed about the portal vein, one on either side of the cannula. The cannula was opened at the same time that the ligatures were tightened. Blood is thus obtained from the desired vein, free from admixture with portal blood. The portal circulation is interrupted for a few seconds, but the pancreatic not at all.

We have collected blood in this manner both before and immediately after painting the pancreas with adrenalin, and analyzed it for sugar. The results are given in Table IV.

The results of these experiments are very uniform. In Experiment 1, the percentage of sugar in the pancreatic blood rose 0.073 per cent in the first three minutes after adrenalin was applied. In Experiment 2, an increase of 0.068 per cent occurs within twenty-five seconds. In Experiment 3, the rise in the first forty-five seconds amounts to 0.032 per cent. In the last experiment, we have continued the collection of blood when the gland was very much congested, and have compared these samples with portions taken at the same time from the femoral artery. The analyses show a continued rise in the sugar percentage, and only a slight difference in the blood from the two sources. We are forced to conclude, therefore, that there is not an increase in the decomposition of sugar in the pancreas antecedent to the rise of sugar in the general circulation, and that the difference observed in the second series, between the reducing power of the blood of the portal vein and that of the femoral artery, is not dependent on processes of this nature in that gland.

Summary of Conclusions.

1. The intraperitoneal injection of adrenalin chloride, as well as the application of that substance to the pancreas, gives rise to a marked increase of sugar in the blood. This hyperglycæmia makes its appearance immediately after the administration, reaches its maximum in from one to three hours, and may continue for over fourteen hours.

2. Simultaneously with hyperglycæmia occurs a decided diminution in the time of extravascular coagulation of the blood. This phenomenon appears to be due also to the application of adrenalin to the pancreas.

3. The cause of this form of hyperglycæmia, as indicated by comparative analysis of the blood flowing to and from the liver, is to be

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attributed, in great part at least, to an increased sugar formation in that organ.

We are indebted to Dr. C. A. Herter for the suggestion of the subject of this work, and for valuable counsel during its progress. We also wish to express our obligation to Mr. William D. Cutter for assistance in a number of the operations.

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A STUDY OF CHRONIC INFECTION AND SUBINFECTION BY THE COLON BACILLUS.*

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II. ON THE DEGENERATION OF THE SPINAL CORD PRODUCED BY REPEATED INJECTIONS OF CULTURES OF A COLON BACILLUS OF LOW VIRULENCE.

Introduction.

In a former communication† I pointed out that a characteristic anemia, in many respects similar to pernicious anemia, can be produced by repeated intravenous injections of living cultures of the colon bacillus, and also drew attention to the fact that this anemia was accompanied, in the later stages, by a progressive degeneration of the spinal cord which gave rise to clinical symptoms such as are frequently met with in the neuritis of pernicious anemia, as well as in certain cases of diffuse degeneration of the spinal cord.

In that series of experiments a number of rabbits were injected, some intravenously, others intra-abdominally, at regular intervals with sublethal doses of living cultures of a colon bacillus which had been isolated from the intestinal mucosa of a rabbit. A progressive anemia was the result, characterized by diminution of the red blood cells, loss of hemoglobin, poikilocytosis, and the presence in the blood of nucleated red cells. This anemia disappeared when the injections of the colon bacillus were discontinued, but promptly returned when the rabbits were again brought under the condition of subinfection.

Besides the condition of the blood, it was observed that when the anemia had reached a certain degree symptoms of nerve degeneration made their appearance, became progressively more severe, and eventually terminated in complete paraplegia.

* Read at the fourth annual meeting of the American Association of Pathologists and Bacteriologists, April 2, 1904, at New York.

† Journ. of Med. Research, Vol. viii, No. 2, pp. 344–360.

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ON THE CLINICAL MANIFESTATIONS OF THIS PROGRESSIVE SPINAL CORD DEGENERATION. — The degree of anemia which it is possible to produce in the rabbit by this method of subinfection is not extreme when compared with that found in certain cases of pernicious anemia, being about twenty-five per cent of the total number of red cells; the hemoglobin was reduced in the same instance to ten per cent of the normal amount.

The first indication that the nervous system had been affected was noted when the red blood cells had been diminished about one-half, and this occurred regularly in all the rabbits when the number of red cells became equal to about fifty per cent of the normal number. The lower lumbar neurones were first affected, causing incoördination and loss of function of the hind legs, which then appeared to be in a condition of spasticity. There was no direct evidence of pain or tenderness, but when compared with normal rabbits it was quite apparent that there was general hyperesthesia of the hind legs of the anemic rabbit. The reflexes were increased and clonus could be readily demonstrated. The rabbits hobbled about in a peculiar manner, but without much difficulty, and otherwise were not inconvenienced by their condition. As the degeneration progressed the reflexes were diminished and finally lost entirely. Spasticity gave place to a flaccid condition, and the muscles of the hind legs became markedly wasted. Moreover, shortly after the reflexes disappeared there was loss of control over the sphincters, which continued until death. The process of degeneration gradually ascended the spinal cord and eventually affected the fore legs, which also became partially paralyzed. The muscles of the trunk were also affected and it is probable that the bulbar centers were becoming involved, because towards the last the rabbits had difficulty in swallowing food, although up to this time the appetite of these rabbits had been fairly good throughout the experiments.

PATHOLOGY. — Two of these rabbits became considerably emaciated before death; the others, however, remained in

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fairly good condition, there being a moderate amount of subcutaneous fat of a yellowish hue. The muscular atrophy of the hind legs was well marked in every instance.

With regard to the internal organs the changes were slight. The alimentary canal, apart from the anemic condition of the mucosa, presented a normal appearance. The liver, usually of a dark earthen color, was but slightly altered in size, being somewhat smaller than normal; the connective tissue in some of the lobules was slightly increased. The nuclei of a large part of the liver cells were swollen and vacuolated; the liver cells contained a large amount of pigment which in the lobule gave the reaction indicating the presence of iron. The kidneys were but slightly affected, there being a moderate degree of cloudy swelling and some proliferation of the cells lining a few of the convoluted tubules. The urine at no time had contained either albumin or sugar. The lungs presented nothing abnormal. The heart was of normal size, paler in color than usual; valves and orifices were normal, and the heart muscle was slightly friable. In some portions the muscle fibers had lost their striation, while in other areas definite fatty degeneration could be made out.

The muscles of the hind legs showed marked loss of substance of many of the muscle cells with a corresponding relative increase of connective tissue; a few of the muscle fibers appeared to be normal, but were mingled with fibers in all stages of degeneration. The nerve terminals in the muscle bundles were not made out satisfactorily. The blood vessels in the intermuscular spaces were somewhat thickened, but did not show amyloid degeneration.

Pathology of the nervous system. — The spinal cord of these rabbits, together with portions of the peripheral nerves, were hardened, partly in Müller's solution and partly in alcohol; the nerve tissue was stained by Pal-Weigert hemotoxylin, Marchi, Nissl, and Van Gieson.

The peripheral nerves, with the exception of some of the fibers of the sciatic nerve, were unchanged. The sciatic nerve when stained by the Pal-Weigert hemotoxylin method,

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showed in some of the fibers a slight degree of myelin sheath degeneration, and when stained by Marchi a few of the fibers appeared to be undergoing a slight degree of fatty degeneration. Fragmentation of the fibers could also be made out, but this may have been due to artifact.

In the spinal cord the changes consist of a myelin sheath degeneration of the fibers in the posterior and postero-lateral columns of the cord, more particularly of the lumbar and dorsal regions, the other portions of the white matter remaining uninvolved. The sections from the upper lumbar and dorsal regions are riddled with vacuoles of varying size, which cannot be regarded as artifacts, because the change is confined entirely to the degenerated areas, and further, these vacuolated areas in sections stained with osmic acid show a marked preponderance of fat, indicative of fatty degeneration. The neuroglia shows little or no tendency to proliferate, and this, together with the destruction of the myelin sheath cells through fatty degeneration, gives to the diseased areas their peculiar sieve-like appearance. In the cervical region the degeneration is less extensive and is confined chiefly to the posterior columns. The ventral portions of the cord throughout are unaffected.

Gray matter. — This is normal except for a certain degree of pigmentation and other changes of some of the ventral horn cells. In some instances the entire cell body is filled with pigment, in others the nuclei have disappeared, possibly as the result of the deposit of pigment. In other instances the nuclei are distorted in shape and occasionally are lacking in distinctiveness of outline. A few cells show an absence of granulation. A large portion of the cells appear to be quite normal; some of the cells of the dorsal horns also show increased pigmentation, the cell bodies being almost completely filled with pigment and the nuclei, when seen, are placed eccentrically and are distorted in outline.

Nerve roots. — The roots, both ventral and dorsal, appear to be normal. The changes just described in the ventral

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horns, whatever may be their functional significance, have not produced in their associated nerve roots organic changes of such a character as to be detected with our present methods of technique.

Blood vessels. — These appeared to be normal, both in the pia and in the cord itself.

CONCLUSION. — These changes in the spinal cord, brought about by the condition of subinfection, the result of repeated injections of the colon bacillus, are of the nature of a diffuse degeneration of the cord confined to the posterior and lateral columns, and most extreme in the lumbar and dorsal regions. It is characterized by a fatty degeneration of the myelin sheath cells of the nerve fibers, little or no increase of neuroglia, definite changes in nerve cell bodies. There is no nerve root degeneration and no abnormality of the blood vessels.

III. ON THE EMPLOYMENT OF THE CULTURE PRODUCTS OF THE COLON BACILLUS IN THE PRODUCTION OF A STATE OF SUBINFECTION.

In order to study the effect of repeated injections of the culture products of the colon bacillus, I have undertaken various experiments upon rabbits, using the same strain of colon bacillus which I had employed in the above research on subinfection. These further experiments are:

(A.) On the effects produced by repeated subcutaneous injections of killed cultures of the colon bacillus.

(B.) On the injection of filtered cultures of the colon bacillus.

(C.) On the action of pepsin and hydrochloric acid upon cultures of the colon bacillus and the effects produced by the injection of the digested bacilli:

(1.) The action of pepsin and hydrochloric acid upon living cultures of the colon bacillus.

(2.) The action of pepsin and hydrochloric acid upon killed cultures of the colon bacillus.

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A. On the effects produced by repeated subcutaneous injections of killed cultures of the colon bacillus. — It is generally stated that the pathological lesions and conditions produced in the animal body by microörganisms are due to the action, upon the tissue cells, of the bacterial toxines which have been elaborated outside of the body, as it were, for example, in the intestinal canal, and being absorbed into the blood and brought into contact with the tissue cells, set up their characteristic pathological conditions. William Hunter[2] has shown that the probable cause of pernicious anemia is the absorption into the blood, under certain conditions, of toxines and other products of bacterial growth from the intestinal canal which, by their presence in the blood, bring about destruction of the red cells and the subsequent production of anemia.

On the other hand, as I have already shown, a characteristic anemia, in many respects similar to pernicious anemia, can be produced by injecting into the circulating blood small, repeated doses of living cultures of the colon bacillus, one of the normal inhabitants of the alimentary tract. The bacilli are thus brought into actual contact with the elements of the blood and the tissue cells in general, and while no doubt many of the bacilli are taken up almost immediately by phagocytes and destroyed, others remain alive, multiply and produce their toxines in close proximity to various tissue cells, which are thereby destroyed. The red corpuscles of the blood are particularly susceptible to the proximity and action of the colon bacillus and rapidly undergo hemolysis. This may be readily demonstrated by adding a few drops of sterile blood to a freshly sown broth culture of the colon bacillus. After a few hours in the incubator at 37° C. many of the red corpuscles will be found to be deprived of their hemoglobin, although the corpuscle remains intact — a shadow cell. This culture of blood and broth when examined with the spectroscope shows the presence of reduced hemoglobin. These, therefore, are some of the effects which are brought about by the presence in the circulating blood of living colon bacilli.

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In order to ascertain whether similar effects could be produced by injecting into the circulating blood the dead bacilli and their culture products, other groups of rabbits were selected and placed under similar conditions to those which were being injected with the living cultures of the colon bacillus.

Group III. — The rabbits of this group were injected subcutaneously at regular intervals with killed broth cultures of the same colon bacillus which was being used in Groups I. and II. These cultures were grown in flasks during twelve days at 37° C. and were then killed by being subjected to a temperature of 55° C. for an hour. They were then tested on three successive days by means of streak cultures upon neutral agar, in order to make sure that they were quite sterile. This being established, the rabbits were injected subcutaneously, at intervals of two days, with gradually increasing doses, commencing with one cubic centimeter. Similar routine examinations of the daily weight, temperature, and condition of the blood of these rabbits were conducted as in Groups I. and II. The injection of these killed cultures did not produce conditions similar to those brought about by the living cultures of colon bacilli.

The immediate effect produced by an injection of these killed cultures was a rise in body temperature of one to three degrees, the maximum being reached about eight hours after the injection had been given; the temperature returned to normal within twenty-four hours. During this febrile state the rabbits were somewhat dull; the respirations and pulse rate were accelerated, and frequently the rabbit fur was damp with perspiration. The appetite of these rabbits was unaffected at any time, and there was a gradual though constant gain in weignt, due to an increase of subcutaneous fat.

The degree of anemia produced by these killed cultures was slight and transient. The reduction in the number of red cells was most rapid during the first month of the experiment and in the most susceptible rabbits amounted to a decrease of 2,000,000 red corpuscles per cubic millimeter. The decrease during the same period in the case of the

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remaining rabbits was 1,000,000 to 1,500,000 per cubic millimeter. The white cells increased 2,000 to 3,000 per cubic millimeter. During the second month of the experiment, although the amount of the killed cultures injected had been gradually increased, there was no further reduction in the number of the red cells. The white cells were but slightly increased in number. The rabbits looked well and continued to gain in weight. During the third month the number of the red cells began to increase, and this increase continued until at the end of six months the blood of these rabbits contained a greater number of red cells than at the commencement of the experiment. The slight degree of leucocytosis continued. The tolerance of these rabbits towards the killed cultures became greatly augmented, until finally a dose of twenty to thirty cubic centimeters produced less constitutional disturbance than did one-half cubic centimeter at the commencement of the experiment.

The gain in weight at the end of six months amounted to from forty to fifty per cent of the original weight, and the healthy, thrifty appearance of the rabbits of this group contrasted strongly with the emaciated and paralytic condition of the rabbits of Groups I. and II.

Nucleated red cells were not present at any time in the blood of these rabbits and there was no poikilocytosis. None of these rabbits died during the experiment nor during the succeeding six months, that is, all were alive and well twelve months after the commencement of the experiment. No subcutaneous abscesses followed the injection of these killed cultures, even in quantities of twenty-five to thirty cubic centimeters.

B. On the injection of filtered cultures of the colon bacillus. Group IV.—Another group of rabbits, under similar conditions of hygiene and routine treatment, were injected with filtered cultures of the colon bacillus.

The cultures were grown in flasks during twelve days at 37° C. and then filtered through porcelain filters until absolutely sterile. The injection of this filtrate was without any

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result in the production of anemia and, though injected in quantities of three cubic centimeters at the commencement of the experiment, produced but little constitutional effect. These rabbits at the end of six months had not gained as much in weight as the rabbits of Group III., and presented scarcely any departure from the control rabbit kept under similar hygienic conditions.

C. On the action of pepsin and hydrochloric acid upon cultures of the colon bacillus and the effects produced by the injection of the digested bacilli.— In order to ascertain the nature of the action of pepsin upon the colon bacillus under conditions approximating those present in the stomach during the process of digestion, I undertook the following experiments:

(1). Cultures of the colon bacillus, such as I was using in the above experiments, varying in age from twenty-four hours to twelve days, were titrated to 0.2 per cent acidity with sterile hydrochloric acid. A quantity of pepsin of high digestive power was then added and the cultures were replaced in the incubator at 37° C. They were allowed to remain for different periods of time, varying from five hours to several days. I found that, although a number of the bacilli in the culture died and became at length more or less disintegrated, there were always a large number of actively motile, living bacilli, probably the younger generations, which persisted indefinitely and rendered it impossible to obtain a sterile culture product by this means. Moreover, the pepsin, at first quite active as a digestive ferment, gradually lost this property, until at length after twenty-four to forty-eight hours it was entirely absent. The pepsin being of the nature of a proteid, it is probable that it was eventually broken down by the colon bacilli. This culture product, being, as it were, a living culture of colon bacilli, together with an increased amount of end products of proteid decomposition, was subsequently injected intravenously into rabbits, but its action in any respect did not differ materially from that of the living cultures employed in Groups I. and II.

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(2.) Killed cultures of the colon bacillus, similar to those used in Group III., were treated with pepsin and hydrochloric acid and placed in the incubator at 37° C. Under the action of the pepsin the capsules of the bacilli were more or less disintegrated and formed a precipitate at the bottom of the flask. This precipitate could be stained with the ordinary dyes, though not so satisfactorily as a preparation of living bacilli after forty-eight hours. This process of digestion was checked by heating the material at 55° C. for an hour.

These digested unfiltered cultures were injected subcutaneously into another group of rabbits. This material proved to be non-toxic, and large quantities could be injected without serious results. It did not produce any decrease in the number or character of the red corpuscles and beyond a slight leucocytosis and a moderate rise in temperature, which occurred within a few hours after each injection had been given, no effects could be observed. It is possible that the pepsin, besides removing the capsules of the bacilli, had destroyed the toxines as well; and, indeed, seeing that these toxines are themselves of the nature of proteids, it is quite probable that they underwent decomposition during the process of digestion and were thus deprived of their toxic properties.

DISCUSSION.

With regard to the etiology of anemia in general and of pernicious anemia in particular, the general opinion seems to be that the condition may be due to a number of causes. William Hunter,[2] in his admirable work on pernicious anemia, has indicated many of these causes and their effects, and is of the opinion that pernicious anemia is no mere aggravation of an ordinary anemia, but a special and distinct variety. It is, moreover, a not very common disease and its causation is probably dependent upon a particular combination of circumstances, the chief factor being, as Professor Adami[3] has pointed out, a condition of latent infection and subinfection by intestinal bacteria, more especially by the colon bacillus.

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Many observers maintain that pernicious anemia is due to the absorption from the intestinal canal of substances produced by bacterial growth, chiefly toxines and the products of proteid decomposition. It may be true that some of these decomposition products are absorbed under certain conditions into the blood, but Vaughan[4] has proven by careful analysis, that the toxine of the colon bacillus remains intracellular and is not found free in the culture medium. It cannot, therefore, be absorbed from the intestine. Certainly in these studies the products of growth and the dead bacteria have not caused the same pronounced changes as have the live bacilli. The live bacilli, however, are absorbed, perhaps not constantly, but under certain conditions, and bring about the state of subinfection which ultimately may give rise to the process of anemia. This condition of subinfection I have imitated by introducing the living cultures of the colon bacillus directly into the general circulation, with the results already mentioned. It is possible that other microörganisms may exert certain effects under similar conditions, and it is more than likely that the several races of the colon bacillus may each produce a characteristic effect upon some or other of the various groups of tissue cells throughout the body, thus producing definite and distinct pathological conditions. Here let me repeat that I in no sense claim to have experimentally reproduced the classical symptoms of pernicious anemia proper. What these observations seem to prove is that an anemia of pronounced character can be developed by these injections of sublethal doses of a colon bacillus, — typical in every respect save that it is of low virulence, — an anemia accompanied by some of the most marked features of pernicious anemia. It is this family likeness, if I may so express it, between the two anemias that would seem definitely to sustain the conclusion that pernicious anemia itself is due to a subinfection, — to the repeated entry into the system of microorganisms which, while they do not proliferate, as in infections proper, nevertheless, by their presence and by the products liberated in their destruction, have an eventual deleterious effect on the tissues.

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This subject of subinfection is worthy of extended research, and if this be conducted along the lines of inquiry into the toxic action of bacterial products when introduced into the general circulation, with coincident study of the action of the living bacteria themselves upon the tissue cells, or as it may very properly be termed, their parasitic action, valuable additions to our present knowledge of the etiology of certain diseases appear to be promised.

It is my purpose to continue this study of subinfection, employing other races of the colon bacillus. The results will be published from time to time.

CONCLUSIONS.

1. In these experiments there has been developed a state of advanced anemia, not quite comparable with any of the classic forms seen in man. In some respects it resembles pernicious anemia, namely, in the very great diminution in the number of erythrocytes, the marked poikilocytosis, and the appearance of nucleated red corpuscles.

2. On the other hand, it differs from pernicious anemia in the fall of the amount of hemoglobin being parallel with the decrease of the red corpuscles; in the absence of a distinct and extensive Quincke's siderosis; in the absence of any clear evidence of inflammatory or other disturbances of the digestive tract, and of well-marked changes in the bone marrow.

3. In the advanced stage of this anemia a diffuse degeneration of the spinal cord was set up, affecting the posterior and lateral columns of the cord, in the lumbar and dorsal regions. This degeneration consisted in a fatty degeneration of the myelin sheaths of the fibers and certain changes, pigmentary, in the nerve cell bodies of the gray matter. The ventral columns of the cord and the blood vessels were unaffected.

4. Similar conditions of anemia and spinal cord degeneration could not be produced by injecting killed cultures of the colon bacillus, nor by filtered cultures. When the living

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cultures were acted upon by pepsin, and injected intravenously, they did not differ materially in their action from the original living cultures.

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The
Journal of Infectious Diseases

FOUNDED BY THE MEMORIAL INSTITUTE FOR INFECTIOUS DISEASES

VOL. I *November 5, 1904* No. 4

THE LIFE-HISTORY OF TRYPANOSOMA LEWISI AND TRYPANOSOMA BRUCEI.*

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* This paper was presented as a thesis for the degree of doctor of philosophy, University of Michigan. Received for publication August 4, 1904.

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INTRODUCTION.

IN connection with the more special work of cultivating trypanosomes various observations bearing upon the life-history of *Tr. Lewisi* and *Tr. Brucei* have been made. These two forms have been widely studied, and their life-cycles in the vertebrate host appear to be well known. The transmission of *Tr. Lewisi* from rat to rat by means of insect parasites, and of *Tr. Brucei* from one animal to another by means of the Tsetse fly, has been established. What changes the protozoa undergo in these insect hosts is an interesting problem which would seem worthy of further investigation. The recent paper of Schaudinn[5] in which he classifies two intracellular hematozoa as *Trypanosomata*, as a result of having found trypanosome-like stages in the insect host, would indicate the existence of an intracellular stage for the generally recognized trypanosomes and to call for proof that this stage has not been overlooked. Such a paper from so eminent an authority is sufficient proof of the uncertainty which still obtains in regard to the complete life-history of these organisms.† Our observations do not touch upon this problem, but have been chiefly confined to the trypanosome in its mammalian host and in artificial culture. It is the purpose of this paper to bring together these observations and correlate them with the previous work upon this subject.

CLASSIFICATION.

Doflein[4] classifies the blood parasite discovered by Lewis[11] in the wild rat of India as follows:

Stem, Protozoa.
Sub-stem, Plasmodroma.
Class, Mastigophora.
Sub-class, Flagellata.
Family, Trypanosomidæ.
Genus, Trypanosoma.
Species, Trypanosoma (Herpetosoma) Lewisi.

The generic identity of the rat trypanosome with that of the frog, *Trypanosoma sanguinis*, Gruby,[6] has been established by Laveran

†Since this paper was written Novy and Mac Neal have reached conclusions, based on experiments with pure cultures of bird trypanosomes, which do not uphold the views of Schaudinn. The results obtained will probably appear in the next number of this JOURNAL.

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and Mesnil,[11] so that the specific name *Trypanosoma Lewisi* may be finally accepted for this organism.

TRYPANOSOMA LEWISI.

For the study of its life-history there are several excellent guides. The pioneer work of Rabinowitsch and Kempner[24] has been followed up by Wasielewski and Senn,[29] and by the researches of Laveran and Mesnil,[12] and others of less note.

According to Lewis, this trypanosome is found only in the blood of wild rats, *Mus decumanus* and *Mus rufescens*. In the blood of the former species it occurs in many parts of the world. In India, Lewis found 29 per cent. of the wild rats infected with it. Carter[2] at Bombay found 12 per cent., and Lingard,[15] likewise in India, found over 30 per cent. of wild rats infected. Crookshank[3] observed the trypanosome in 25 per cent. of London rats examined by him. R. Koch[8] at Daressalam found 10 infected rats out of 24 examined (41.7 per cent.). Rabinowitsch and Kempner[24] reported 41.8 per cent. at Berlin. At Paris, Laveran and Mesnil[12] found it in 2 out of 43 rats examined (4.7 per cent.). Voges[28] in South America examined numerous rats with negative results. Rouget[26] at Constantine in Algeria failed to find trypanosomes in wild rats. They were found also by Dutton at Bathurst, Senegambia; by Calmette at Lille, and by Buard at Bordeaux. Francis[5] examined 60 wild rats from different parts of Washington, D. C., without finding the parasite. They have been found at Philadelphia, Detroit, Lincoln, San Francisco, and Manila. Of 107 rats caught in Ann Arbor 5 were found infected (4.67 per cent.).[18] These five rats were caught at different times in the same barn. Lately another focus of the infection has been found here in a certain cellar. It is interesting to note in this connection that Lewis in his original paper called attention to the focal character of the infection. Others seem not to have observed this fact. We may conclude from the foregoing that the distribution of *Tr. Lewisi* is as wide as that of its host.

It should be noted, however, that *Tr. Lewisi* is by no means the only trypanosome to be found in the blood of the wild rat. Thus in India it is said that the rats harbor the surra trypanosome.

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This may or may not be true, for the only fact established thus far goes to show that the trypanosome present is pathogenic for other animals than the rat, in which respect it differs from *Tr. Lewisi*. The possibility remains that several species of trypanosomes may be present in the wild rat. No identification can be considered as complete without a full study of the morphological, structural, pathogenic, and above all the cultural characteristics.

Trypanosoma Lewisi does not multiply indefinitely in the rat, but passes through a short period of multiplication which is followed by a relatively long stage in which the organism persists, but without any increase in numbers; further multiplication taking place only in a new host. In a few instances, particularly in young animals, multiplication of the parasite continues until the death of the rat results. The fact that rats may die of this infection was first observed by Wasielewski and by Jürgens.[7] In our first paper[16] we have recorded several deaths from this cause. In general, however, the host survives the developmental stage, and the trypanosomes then persist in the blood without multiplying for months, and gradually diminish in number and disappear. In one white rat the parasites were still numerous nine months after inoculation. This stage corresponds to that met with in Texas fever and in human tick fever, where the protozoa may persist in the blood of the animal for months after its recovery.

Transmission to a new host can take place by the bites of fleas and lice. Rabinowitsch and Kempner[24] report one case of transmission by fleas. As stated in our first paper (*loc. cit.*), we have frequently observed trypanosomes in the stomach of lice fed upon infected white rats. In one instance several such lice were transferred to a fresh young rat (February 4, 1904). Positive infection resulted, numerous trypanosomes being found in the blood after fourteen days and persisting for five weeks. Microscopical examination of these lice, living or crushed, frequently reveals the parasite, but no stages of development as yet have been made out. It seems probable that the louse merely carries the protozoon from one animal to the other.

Experimental inoculation is carried out by injecting infected blood intraperitoneally or subcutaneously. Infection usually

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results in full-grown white rats, and almost invariably in young rats. For old rats intraperitoneal injection of a large dose is often necessary to produce the desired result. The multiplication at the point of injection is of doubtful importance. It is certain that the trypanosomes quickly gain entrance to the blood stream, and that the real multiplication occurs there. They may be readily found in the blood four hours after intraperitoneal injection of a large dose, and such individuals resemble in every respect the trypanosomes injected. If dividing forms are present in the injected material, they continue to divide, and a rich blood infection is quickly produced. If, on the other hand, only adult trypanosomes are injected, the first dividing forms are found on the fourth day, and the cessation of division occurs correspondingly later.

In the adult stage (Plate III, Fig. 1) *Tr. Lewisi* is 27–28 μ long, whip included, and $1\frac{1}{2}$–2 μ wide. Unlike the dividing forms, the whole body takes part in the undulating movement. By the Romanowsky method or its various modifications, this stage stains much less readily than do the division forms. On comparison, the protoplasm stains a much lighter color and the mottling with colorless areas is more apparent. The posterior tip in particular is often feebly stained. This is in marked contrast to the readiness with which the division forms take the dye. In well-stained preparations of the adult stage the parasites are all of the same type. The length is very constant.

The smaller chromatin body, which has been designated by several names, such as basal body, micronucleus, centrosome, and blepharoplast, is situated about 3 or 4 μ from the sharp posterior tip. It measures 1 μ in length by $\frac{2}{3}$ μ in width, and is placed transversely, extending across the entire width of the cell at this point. The flagellum at times appears to originate in this body, but the study of more favorable specimens clearly shows an achromatic space between them, as has been pointed out by Laveran and Mesnil. That there is an intimate and firm connection, however, cannot be doubted, for these structures often remain attached together when all the rest of the cell has disintegrated. The flagellum extends in a rather smooth curve, along the convex

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border of the parasite, supporting the undulating membrane, and is prolonged about 7 μ beyond the anterior tip of the body as a free whip.

At approximately 11 μ anterior to the blepharoplast is found the nucleus; this also is oval in form, measuring $2\frac{1}{2}$ μ by $1\frac{1}{2}$ μ, with the long axis longitudinal. In structure it presents a chromatin network in a lighter nucleoplasm. At times a grouping of the chromatin into twelve chromosomes is apparent.

After injection into a new host, this adult form is found without any perceptible change for three days. Whether in every case it first enlarges and then divides, or begins division at once to form the small type of trypanosome, is not certain, as the large and small forms appear in the peripheral blood simultaneously on the fourth day. It seems probable, however, that enlargement precedes division. This is the case in culture. We may assume that the injected trypanosome first enlarges and tends, to some extent, to lodge in the internal blood capillaries, whence its smaller progeny can more readily escape. Aided by the constant high internal temperature, the development of such lodged forms would proceed a little more rapidly than in those freely circulating in the blood stream. An examination of streak preparations of the internal organs of rats at the end of the third day after inoculation often shows a larger proportion of developmental forms than does the blood, although the number of trypanosomes present is not greater than the blood content would account for. This has been noticed more particularly in the case of the adrenal. But, be that as it may, the fact remains that the large and the small forms appear in the peripheral blood of the tail at the same time.

In their pioneer work upon this subject, Rabinowitsch and Kempner[24] described three modes of multiplication: longitudinal division, transverse division, and segmentation. Wasielewski and Senn,[29] by study of living preparations and by employing an improved staining method, were able to show that the differences in arrangement of nuclei of the dividing cells, which was for the former workers a characteristic distinction between longitudinal and transverse division, were due to manipulation. Likewise they

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showed conclusively that the characteristic feature of the segmentation, namely, "that division was initiated only after loss of the undulating membrane and flagellum and the rounding up of the parasite," did not exist in reality, and that Rabinowitsch and Kempner had been deceived by the stain employed. They thus established unequal longitudinal division as the only method of multiplication of *Tr. Lewisi*, and showed that apparent differences were nonessential variations of this one process.

Laveran and Mesnil[12] recognized two modes of multiplication: unequal longitudinal division (Group *a*), and rosette formation (Group *b*)—a simultaneous division into several coequal daughter-cells, no mother-cell being distinguishable. They were inclined to regard this latter as a variety of longitudinal division rather than as a segmentation.

Rabinowitsch and Kempner[25] in a later contribution accept Wasielewski and Senn's corrections for the most part, but still maintain that the rosette formation is a distinct mode of multiplication, and that the mother-cell is not to be recognized in such rosettes.

Our observations in regard to the division of *Tr. Lewisi* are similar to those of Wasielewski and Senn. The characteristic feature of the Group *b* of Laveran and Mesnil does not exist in reality. Unbroken multiplication rosettes show the mother-cell (Plate XIV, Fig. 2), and even when broken up, the division of the separate elements continuing, is still essentially unequal, as appears, in the same figure. It is only more difficult to distinguish between whips which have formed in rapid succession than between one of these and the old, full-grown whip. The essential inequality is always there, as must follow from the fact, as will be shown, that the whip multiplies, not by division, but by new formation.

Beginning then with the adult stage, we may say that the protoplasmic body increases in length, breadth, and thickness, and in staining power (Plate XIII, Fig. 3). The flagellum does not elongate, but becomes thicker and more readily stainable. The blepharoplast elongates slightly, the nucleus becomes larger and less definitely outlined, and its chromatin more loosely arranged,

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so that often the nucleus is almost concealed by the densely staining protoplasm. The blepharoplast and the nucleus approach each other. In a living preparation this form is seen as a wide stiff spindle drawn along by the rapid vibratory motion of its relatively short flagellum. The motion is markedly different from that of the adult type. It may be worth noting that the whip, though relatively short, is by actual measurement of the same length as that of the adult trypanosome.

Division begins in the blepharoplast and flagellum, less frequently in the nucleus, and then only in the later stages of infection. It is frequently possible to make out the so-called division of the flagellum before that of the elongated blepharoplast is apparent. The split appears to extend along the whip to about its middle, at which point the daughter-whip breaks out to one side and is shorter than the other. The flagellum really does not split, but the shorter whip grows anew from a base near the blepharoplast (Plate XIII, Fig. 3). This fact is often apparent in blood stains, and has been proved in the case of *Tr. Brucei* by dissolving away the sheath of the flagella by alkaline sodium phosphate, in which case the short new whip is seen free with a pointed end, as in Plate XV, Fig. 4. In general the new flagellum is in relation with the more posterior of the two separating daughter-blepharoplasts. The nucleus divides directly.

The division of the protoplasm is assisted by the lashing of the free flagella. The two resulting cells remain attached longest at their posterior tips. Each daughter-cell proceeds to further division without waiting to enlarge, for an undetermined number of times, and the resulting cells are very small, 10–15 μ in length or even smaller. The larger one which retains the old whip usually proceeds to its second division more rapidly than the other.

The small cells which result from these repeated divisions (Plate XIV, Fig. 7) possess all the structure of the adult trypanosome. The blepharoplast and nucleus lie close together, but rapidly separate as the cell elongates. The undulating membrane is a rather rudimentary fold of the outer protoplasm, which elongates as the chromatin bodies separate. Various stages in the growth of these small forms are seen in Plate XIV, Fig. 3, which also

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serves to illustrate the variety of size which *Tr. Lewisi* presents during its developmental stage in the rat.

In culture on blood agar *Tr. Lewisi* may give rise to much smaller forms. Cells with a body-length of 5 or 6 μ are common (Plate XIV, Fig. 8), and smaller forms, 2 or 3 μ in length, have been frequently seen. Such cultures after passage through a Berkefeld filter still infect rats.

In the first days of the developmental stage the daughter-cells separate as soon as formed, but later, about the sixth to the eighth day, they tend to remain attached at their posterior tips while continuing to divide, thus forming rosettes of four, eight, sixteen, or even more cells. The eight-cell rosettes are quite common (Plate XIV, Fig. 2). Their formation is due to the increasing difficulty in division, which is associated with a gradually increasing stickiness of the trypanosome body and indicates a beginning antagonistic action on the part of the host. Finally, about the ninth or tenth day, dividing forms are no longer found, the trypanosomes assuming the slender form of the adult type.

At this time occurs a more or less marked agglutination of the trypanosomes. The posterior tips become very sticky and readily attach themselves to blood corpuscles or to each other. Two trypanosomes touching at their posterior tips stick and remain in contact in spite of the apparent effort to free themselves. Others catch by their posterior tips at or near this junction, and thus agglutinations of a hundred or more cells may be formed. In some rats all the trypanosomes are gathered into such agglutination rosettes at one time. In most cases, however, only a small number of the parasites are found agglutinated at any one examination. After a day or two the trypanosomes are again single and often as numerous as before, but gradually decrease till finally after several weeks they disappear entirely. In rare cases this agglutination results in the sudden disappearance of all the parasites. The phenomenon may be considered as a peculiar reaction of the trypanosomes to an immunity on the part of the host. It is less marked in the more susceptible young rats.

The contact of the two agglutinated trypanosomes is not merely at the extreme posterior tips, as described by Laveran and Mesnil,[12]

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but most frequently the tips overlap each other somewhat (as in Plate XVI, Fig. 5), resembling the figures which Plimmer and Bradford[21] have given as conjugations of *Tr. Brucei*. Changes in the smaller chromatin body, which they call the micronucleus, are difficult to make out and their interpretation is necessarily doubtful. At this stage and under high magnification, this body always presents a bilobed or hour-glass appearance. In stains of blood containing the agglutination rosettes many of the trypanosomes are separated in the act of spreading the film. In some instances fragmentation of the blepharoplast is distinct and certain, two or three chromatin bodies of unequal size being found at the posterior tip. We shall return to these problematical observations later under the subject of conjugation.

As stated above, the trypanosomes after agglutination separate again, as a rule, and continue single in the blood for some time. These adult forms are distinctly different from the dividing forms. The constant size, the feeble staining power of the protoplasm, and the compact and sharply outlined nucleus are in strong contrast with the features presented by the dividing forms and mark this as a distinct type of the organism. In the later stages of infection this adult type may be found, though rarely, undergoing a slow and apparently greatly retarded longitudinal division. We have observed this condition as late as the one hundred and tenth day of the infection. The trypanosomes in this case are larger than the others and contain two nuclei, one anterior to the other. They are quite different in appearance from the dividing cells of the developmental stage.

In the final destruction of these trypanosomes Laveran and Mesnil assign phagocytosis the chief rôle. By injecting trypanosomes into the peritoneal cavity of guinea pigs immunized to this organism, and subsequently examining the peritoneal fluid, they were able to find various stages of ingestion of the protozoa by the leucocytes. We have attempted to confirm this by a similar procedure. Instead of a phagocytosis, however, we could find evidence only of the immobilization and gradual solution of the trypanosomes in the peritoneal fluid. Stains made from time to time showed the protoplasm of the parasites paler than before,

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until finally it seemed to have dissolved. The process appeared to be quite analogous to that met with in the well-known Pfeiffer's phenomenon. In other words, the trypanosomes disappear as the result of the presence and action of cytolytic agents rather than by phagocytosis.

CULTURE OF TRYPANOSOMA LEWISI.

The development of *Tr. Lewisi* may be followed a little more closely *in vitro* than in the living host, but the study is complicated by the presence of degenerating and dying forms. The difficulty in obtaining good stains offers another serious obstacle. The great advantage lies in the fact that the changes which take place in the living cell may be watched under the microscope. The Ranvier slide is the most satisfactory for this purpose, as it gives a thin layer between slide and cover, provides some air supply, prevents desiccation, and may be rendered free from contaminating organisms. Obviously very thin cover-glasses should be employed, so as to permit the use of the oil-immersion objective. The slide and cover-glass are first sterilized by flaming, then a ring of vaseline is applied around the outer edge of the circular well. A loopful or two of the fresh condensation fluid from a blood-agar tube is then placed in the center of the slide, after which a loopful of the culture is added, and the whole is covered with the sterile cover-glass. The study of such preparations was made in a warm room, kept at a constant temperature of 30° C. Under these conditions the trypanosomes in the preparations remain alive and can be kept under observation for two or three days.

A tube of the proper blood agar,[18] inoculated with a drop of blood rich in adult forms of *Tr. Lewisi*, is capped and set aside at 30°. From time to time a loopful of the condensation fluid is removed for study. Within a few hours the trypanosomes are found agglutinated into small bunches of two, four, or more cells, all of which are very actively motile. No further change is apparent until the third day. Then dividing forms are found, large and small individuals and rosettes of four to one hundred cells.

In from three to four weeks the culture becomes quite rich, and various peculiar forms are met with, particularly round granular bodies of different sizes, whose formation may be watched under the microscope. They result from the contraction and rounding of an active long form, motion diminishing at the same time. Frequently two or more trypanosomes lying near each other coalesce to form one large sphere from which a corresponding number of whips extend out in various directions and lash feebly for some time. Repeated observation of such forms under the microscope has shown that they eventually lose all motion. These motionless round bodies might be considered as fertilized cysts, or something of that nature, from their method of formation, were it not for the negative experiments in cultivation and inoculation. Thus, repeated transplantation of different cultures rich in these forms, but free from motile trypanosomes, has always failed to produce another culture. Again, inoculation of animals with such material has yielded equally negative results. They must therefore be considered as dead and degenerated forms.

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After growth for a year or so on artificial media the trypanosome seems to adapt itself to the new conditions. Frequently tubes are found in which veritable colonies can be seen upon the surface of the blood agar above the level of the condensation fluid. These are moist, raised, and glistening white. At first they were mistaken for bacterial colonies, but microscopic examination showed a mass of active trypanosomes in various stages of growth and degeneration.

CELL-DIVISION OF TR. LEWISI IN CULTURE.

A Ranvier slide preparation was made by mixing one loopful of an actively growing culture with two loops of fresh condensation fluid from a blood-agar tube, and studied in the warm room at a constant temperature of 30° C. A careful survey of the whole slide was first made by means of a mechanical stage, and the location of probable divisional forms noted. Division is best observed in the small rosettes of four cells. These have practically no progressive motion, and yet are simple enough for each cell to be clearly seen and followed throughout. After going over the whole slide a certain specimen was selected and kept under constant observation, hasty sketches being made from time to time as changes were observed.

In one experiment a rosette of five cells was selected (Plate I, Fig. 1). The cells in this case possessed little motion and were attached together loosely at their central tips. Sometimes a cell would appear entirely free for a time and later become attached again. All of the changes were slow enough to be easily followed, and there was no tendency in the group under observation to move any distance. A definite whip could be made out only on one cell, the largest. The others appeared to have no whips, or at most a short stub. They possessed a slow swaying motion. When observation was begun at 8:25 A. M., one cell (Fig. 1 *a*), was rounded and an indication of a division line was already present. A second cell, *c*, was also somewhat shortened. Five minutes later the division line in *a* was more certain and a transparent line had appeared in *c*. Five minutes later, at 8:35 A. M., the two parts of *a* had separated so as to leave no doubt, *c* was certainly dividing, and another cell, *d*, was growing thicker and shorter. The next five sketches were made at intervals of three minutes. At 8:41 A. M. (Fig. 5) the two daughter-cells of *a* were lengthening, *c* was completely divided, and *d* showed a faint division line. The fourth cell, *e*, was beginning to shorten and thicken. At 8:55 A. M. (Fig. 9) the three cells, *c*, *d*, and *e*, were all dividing, and the two daughter-cells of *a* had lengthened and become quite actively motile. The next sketch (Fig. 10), made fifteen minutes later, at 9:10 A. M., indicates the altered position due to their activity. At 9:39 A. M. (Fig. 12) the divisions were completed, and the rosette, now consisting of nine cells, was being actively twisted about by the increasing motion of its parts. The motion became so confusing that it was impossible to follow any one cell with certainty. Three hours later, however, it was possible to count at least twelve — sometimes there seemed to be fourteen — cells in the rosette. It appears, therefore, from this that in cultures the *Tr. Lewisi* may divide in an hour and proceed to a second division within four hours. Multiplication in the body must be more rapid than this, for here we were working with a temperature of only 30° C., not to mention other unfavorable conditions.

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The position of the flagella in these rosettes was a puzzle for a long time. They are not to be seen on the peripheral ends of the cells except in rare cases, and then only in the very small rosettes. Rarely they can be seen directed centrally in the living preparation, and the peculiar motility of the cells, as if they were attached to each other by narrow waving stalks, suggests this position. However, the center of a rosette is not distinctly seen in the living preparation, and one cannot be certain that the whips are there. Opposed to this is the undoubted fact that the dividing rosettes in the living blood have their flagella on the periphery. To settle this point it was necessary to stain the whips, which was accomplished by the use of strong Romanowsky-Nocht mixtures.[19] The resulting preparations are somewhat dirty in appearance, but they show conclusively that the whips are central in direction (Plate XIV, Fig. 4). The nucleus and blepharoplast are seen close together near the center of the cell. The flagellum is heavily stained and extends from near the smaller chromatin body toward the center of the rosette, where it is attached to the whips of the other cells.* We may consider such a mass to arise by successive divisions of a single cell, the new flagellum remaining attached to or entangled with the old, while the cell-body has completely divided. It is interesting to note in this connection that Schaudinn[27] has observed rosettes with centrally directed whips in the mosquitoes infected with the halteridium, *Hemoproteus noctuæ*. These he interpreted as agglutinations.

The cell-division is not exactly equal. One daughter-cell always projects slightly beyond the other during division, and when they have separated there is a difference in size—often very slight, however. This inequality is best seen in a single dividing cell which has become caught in some way. Such a cell which was found adhering to the cover-glass in a Ranvier preparation, with only its whip free and moving, was carefully watched. When first observed, the cell-body was oval in form and measured 9 by $4\frac{1}{2}$ μ. It shortened a little more, and in the

*In the recent publication of Laveran and Mesnil (*Trypanosomes et Tryponosomiases*, Paris, June, 1904, p. 77), which appeared since this paper was written, the authors describe and illustrate this rosette formation.

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course of fifteen minutes a second short whip was observed at the base of the old one, and a division line was apparent in the anterior half of the body. Here on account of the fixed position of the object the inequality of the two daughter-cells was easily seen. Moreover, it is a fact that the difference in size is really more marked in the case of a dividing large free cell than is the case for the smaller cells composing a rosette. After another thirty minutes' observation the division was completed except for the attachment at the posterior tips.

Attempts to carry out similar observations upon dividing forms taken from the living host have failed. The change from living blood to blood-agar condensation fluid under a cover-glass put a sudden stop to all division. Cells caught in the very act of division made no visible progress, although the preparations remained alive for two days. Jürgens,[7] however, appears to have demonstrated that there is an actual increase in numbers in cover-glass preparations.

TRYPANOSOMA BRUCEI.

The etiological factor in the Tsetse-fly disease or Nagana of Africa was discovered in 1895 by Bruce[1] in Zululand. He considered the parasite as either identical with or closely resembling that observed by Evans in India. Kanthack, Durham, and Blandford[9] in their report upon this disease give a preliminary description of the protozoon. Plimmer and Bradford[26] in their preliminary note named the organism *Trypanosoma Brucei*. They outlined a very complicated life-history, including conjugation, formation of amœboid bodies, etc., besides multiplication by longitudinal and transverse fission. Laveran and Mesnil[10] first clearly recognized the longitudinal division of the parasite and established it as the only method of multiplication in the mammalian host. They[13] regard the conjugation described by Plimmer and Bradford as merely an agglutination phenomenon, and failed to find such agglutination in freshly drawn Nagana blood, but only after it had been some time out of the body. When the trypanosome blood was mixed with physiological salt solution faintly acidulated with acetic acid, they observed the rapid formation of agglutinations and their disintegration into granular

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dead material. On the other hand, weak solution of soda prevented agglutination. Plimmer and Bradford[21] in a later report confirm the division process as described by the French workers, abandon their transverse division, but still cling to their plasmodial and amœboid types. In this later paper they also describe and figure two special types of the organism—the large hyaline trypanosome, with feebly staining protoplasm and sharply outlined nucleus (Plate XVII, Fig. 2), and the very granular type with its protoplasm full of deeply staining granules (Plate XVII, Fig. 1). All these investigators used trypanosomes descended from those brought to England from Zululand in a living dog by Dr. Waghorn in 1896.

Martini[17] found *Tr. Brucei* in the blood of a Barba pony brought from Togo to the zoölogical garden at Berlin. Employing this material, he has confirmed the multiplication stages described by Laveran and Mesnil.

The virus which we have employed was obtained by Dr. Novy through the kindness of Dr. Thomas, of the Liverpool School of Tropical Medicine, and is the same as that used by the English and French workers. It has served for the cultivation experiments elsewhere described.[19]

In rats and mice this parasite produces death in from two to ten days, according to the amount of infective material injected. Development appears to proceed uninterruptedly, as a rule, from the time of inoculation until the blood is literally swarming with the protozoa, when death occurs. Sometimes there is an interruption to the progress of the disease. The rat, rarely the mouse, sometimes acquires a resistance, and the parasites actually decrease in number. This is only temporary, however; the vigorous multiplication soon returns and death invariably results. In these cases the disease is somewhat longer, lasting twelve to fifteen days.

In guinea pigs and rabbits the resistance is usually more marked. The parasites may become very numerous, and then diminish until the microscope no longer reveals their presence for long periods. They may thus increase and diminish in number several times. However, death always finally results. Our

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guinea pigs have died in from ten to sixty days, rabbits in from twenty to forty days.

We have observed only one type of multiplication, unequal longitudinal division. There is a preliminary enlargement, so that the dividing cell is both longer and thicker than the average. The division process begins in the blepharoplast and flagellum, being apparent first in the latter, as a rule. The division of *Tr. Brucei* is essentially the same as that of *Tr. Lewisi.* The great apparent difference is due to three things. First, *Tr. Brucei* is continually dividing, growth and multiplication going hand in hand; as soon as a cell gets a little larger than the average it divides into two. In the rat trypanosome, on the other hand, the cell grows very large before division is initiated. Then successive divisions take place rapidly, growth fails to keep pace, and hence very small forms result. Second, the Nagana parasite does not shorten and lose its motion during division to any such extent as is seen in the rat parasite. Lastly, there is no sticking together of the daughter-cells to form multiplication rosettes of *Tr. Brucei.*

As to whether the flagellum really splits into two, for part or the whole of its length, or the new whip just grows out with its peripheral end closely applied to the old one, is hard to decide from the ordinary preparations. In stains of Nagana blood it appears actually to split, the forking of the old flagellum being very distinct. According to Laveran and Mesnil,[10] the whip splits to the end of the undulating membrane and at times throughout its whole length. During the course of a series of experiments to test the effect of various salt solutions upon *Tr. Brucei* certain observations were made which throw an important light upon this question. One-fourth of a cubic centimeter of a molecular solution of the normal sodium phosphate was added to 0.75 c.c. of defibrinated rabbit's blood. To this mixture a drop of freshly drawn defibrinated rat blood rich in *Tr. Brucei* was added. After five minutes, microscopic examination showed many trypanosomes with the actively motile whips separated from the body except for their attachment near the blepharoplast. At the end of thirty minutes stained preparations were made, and it is in these that the true relations of the new whip formation seem to have been

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found. The alkaline phosphate dissolves away the undulating membrane which sheathes the flagellum, and this is then set free. The early stages of division in which the flagellum appears to be branched near the blepharoplast, present in these preparations two entirely separate flagella, one of which is very short with a tapering tip (Plate XV, Fig. 4). It would seem, therefore, that the division of the old whip is apparent and not real, and that its sheath forms a path of least resistance for the new whip to grow out into. The new flagellum is formed entirely anew from the blepharoplast, and not by a division of the original whip, as has been heretofore supposed. This corresponds to the observations which have been made on the flagellum formation in the case of *Tr. Lewisi.*

As the new flagellum grows the blepharoplasts separate, the one in relation with it moving posterior to the other, as if the active whip growth pushed its base backward and its tip forward at the same time. The indefinitely outlined nucleus divides directly into anterior and posterior portions. The separation of the flagella from each other produces two undulating membranes which unite at the point of contact of the whips. As this point is pushed farther forward, the two undulating membranes come to occupy the opposite lateral borders of the cell, and a lighter line of thinner protoplasm appears running down the middle between them (Plate XV, Fig. 2). Such cells are often seen in the living preparation. The final division of the protoplasm takes place along this lighter line in such a way that the posterior tip of the mother-cell goes with the new whip and the greater part of the anterior protoplasm with the old whip. As the connecting band between the daughter-cells weakens they tend to slip on each other, the posterior one becoming more posterior, and in this way the cells separate from each other, the complete division being facilitated by the actively motile flagella..

Multiple divisions are not uncommon, three blepharoplasts, whips, and nuclei being present in one large cell at the same time. Simultaneous division into four is rare. As a rule, the daughter-cells separate and enlarge before dividing again, so that there is not the remarkable difference in size seen in the case of *Tr. Lewisi.*

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When the protozoa are numerous in the blood three, more or less distinct, types can be recognized by the variation in the amount of granulation. The first or ordinary type presents a narrow body with a large elongated oval nucleus occupying the whole width of the body. The outline of the nucleus is indefinite. The protoplasm stains deeply and contains granules which stain like the nucleus or somewhat darker. These are found in the protoplasm anterior to the nucleus, few posterior to it.

Far less numerous are the larger faintly staining forms (Plate XVII, Fig. 2). In these the protoplasm is free from granules and stains a clear pale blue. The nuclei are compact, round, sharply outlined, and do not occupy the whole width of the cell. In staining character they bear a suggestive resemblance to the rat trypanosomes undergoing solution in the peritoneal fluid of the immunized guinea pig. If stained preparations are made from the blood of a Nagana mouse at intervals before and after death, the stages of disintegration of the protozoa may be studied. The nucleus of the trypanosome, dying under these conditions, becomes spherical; its protoplasm becomes progressively more feebly staining, and finally dissolves, leaving the round nucleus by the side of the flagellum. The blepharoplast remains attached to the latter. From this it would seem that the large hyaline forms of Plimmer and Bradford are really dying and dissolving in the living blood.

The third or very granular type is somewhat more rare, as a rule. Plimmer and Bradford at first considered them to be trypanosomes which had just completed the act of conjugation, but in their later paper they are inclined to question the correctness of this view. Thus far there is no very definite evidence in regard to their origin or meaning. It seems probable that some of them are degenerating forms as somewhat similar cells are found in old, dying cultures.

Although not so marked as in the case of *Tr. Lewisi*, yet agglutination of the Nagana trypanosome does occur. Collections of from two to five cells are readily found in the freshly drawn blood of the unusually resistant rats previously mentioned, this phenomenon being associated with a diminution in the number of

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parasites present. A similar agglutination is also associated with the reduction in number of the parasites in guinea pigs.

Outside the body, agglutinations readily form, and this tendency is much increased by the presence of acids or acid salts. Laveran and Mesnil noted the marked effect of minute additions of acetic acid. Similar results are obtained with sodium hydrogen sulphate and sodium metaphosphate. Stained preparations of agglutinated material show peculiar changes in the attached posterior ends of the trypanosomes, especially interesting being the appearance of the blepharoplast. These will be discussed later under the head of conjugation.

CULTURE OF TR. BRUCEI.

The Nagana parasite may be grown *in vitro* by a procedure analogous to that used for the rat trypanosome.[18] The results obtained have been published in two papers.[19] A somewhat detailed discussion of the proper medium was given at the last meeting of the Michigan Academy of Science.[16] In general, it may be said that the Nagana trypanosome is more exacting in its requirements, and that its cultivation is more difficult than in the case of the rat parasite. For isolation from the living animal a nutrient agar of the following composition is prepared:

Extractives of 125 g. chopped beef in distilled water	1000 c.c.
Agar	20 g.
Pepton	20 g.
Common salt	5 g.
Normal solution Na_2CO_3	10 c.c.

The agar thus prepared is distributed into tubes or small Erlenmeyer flasks and sterilized in the autoclave. To one volume of the sterile melted agar, cooled to about 60° C., two volumes of naturally sterile defibrinated rabbit's blood are added, thoroughly mixed, and allowed to solidify. Tubes are allowed to solidify in the inclined position; flasks upright. Into the small amount of liquid which collects upon the surface of the blood agar, pure Nagana blood is inoculated, and here development takes place.

Examination in a few hours will show many small agglutinations of from two to six cells, many trypanosomes, however, remaining single. Careful daily microscopic examinations for the next fifteen days may show various forms and changes leading to the death of the trypanosomes. The formation of round bodies, the *mis en-boules* of Laveran and Mesnil, may be readily followed. The body of a trypanosome contracts into a spherical mass with its whip extending out and retaining a feeble motion for some time. Frequently two or more cells lying near each other are seen to coalesce into a large sphere with a corresponding number of whips. That these are death changes is indicated by the failure of such round bodies to infect animals or to give rise to another culture when transplanted to fresh medium. There

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is a steady diminution in the number of protozoa until about the tenth to fifteenth day, when it is often an endless search to find one under the microscope. The disappearance may be final and the attempted culture a failure. In many cases they reappear between the twentieth and thirtieth day, and can then be successfully transplanted. In the most favorable first generations the trypanosomes can easily be found microscopically and an increase in number is then apparent as early as the tenth day. Transplantation from the first generation has been successful when made as early as the fourteenth day and as late as the fifty-second day. After an active multiplication of from four to fifteen days the number again diminishes, and the protozoa disappear finally at about the fiftieth day, often earlier.

For the succeeding cultures inoculated from this first generation, a blood agar with four times as much meat extractives is employed. Upon this the growth is more rapid and abundant, although subsequent degeneration and death is also hastened. The transplantation is performed by transferring a drop of the condensation fluid by means of a sterile Pasteur pipette. The cultures may be grown at room temperature, but a constant temperature of 25° C. is more satisfactory.

DIVISION OF TR. BRUCEI.

Ranvier slide preparations of a growing culture of *Tr. Brucei* were made and studied in the warm room in the same way as in the case of the rat trypanosome culture. Here the study was more difficult, this trypanosome being much more sensitive and readily ceasing all activity under the conditions necessary for the experiment. The small rosettes, which in the case of *Tr. Lewisi* are quiet enough for observation, are here so active that the slide must be continually moved to follow them. The preliminary shortening of the cell before division is not apparent, and for that reason it is more difficult to select suitable cells for observation. It was necessary to select a cell, largely at random, and to watch it continuously for any change. Naturally, large cells might be expected to divide sooner than small ones. As the result of a week spent in the warm room cell-division was twice observed, once in a cell of a small actively traveling rosette, and once in a large single cell attached by its posterior tip to a mass of blood corpuscles.

A slide preparation of an actively growing culture was made at 8:55 A. M. After going over the whole preparation, using the mechanical stage, a rosette of three cells was selected as the most promising, because one of the cells composing it was somewhat larger than either of the others. The large cell, *a*, was overlapped for its posterior one-third by the posterior end of cell *b*, the whips extending free in opposite directions. Cell *c* was wound spirally around the junction of *a* and *b*, its whip extending laterally. The large cell by its active whip overcame the exertions of cell *b* in the other direction, so that the whole rosette traveled constantly in its direction; the third cell, *c*, by its position produced a constant spiral rotation of the rosette. Observation was begun at 9:41 A. M. At 2 P. M. a second whip was seen on cell *a*, very short and close to the old one, although its presence was not certain. At 3 P. M. there was no longer any doubt. A new whip was there and very active. In another half-hour it was much larger and the division had

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extended into the cell-body for some distance. The split progressed somewhat farther, but at 4:30 P. M. the motility decreased, and the outlines between the cells became indistinct, and at 5 P. M. all three cells had coalesced into one spherical body, one of the whips, that belonging to cell *b*, still retaining slight activity.

The second observation of division was made upon a single cell (Plate II, Fig. 1). The slide was prepared at 9:30 A. M. The cell was selected on account of its large size and relatively fixed position, and the observation was begun at 10:30 A. M. It was then an unusually thick cell with a long cylindrical posterior tip and a very active flagellum. By its posterior tip it was attached to a mass of blood corpuscles, so there was no progressive motion. The cell is represented in Plate II, Fig. 1. At 6 P. M. a second whip seemed at times to be present on one side of the cell, and at 8:55 P. M. its presence was no longer in doubt (Fig. 2). At 8:52 the next morning this whip was longer, and the division of the cell-body could be indefinitely made out by focusing (Fig. 3). An hour later the outline of two cells was distinct and certain, the smaller one lying beneath and anterior to the larger. Both were very active. From the large size of the upper cell a second division was expected to occur soon. The specimen was therefore kept under observation, but all activity ceased at 6 P. M.

A careful examination of the rosettes of *Tr. Brucei* in culture shows them to be made up of cells stuck together in this relation —that is, by their adjacent sides—one cell being slipped forward on the other, as it were. We can conceive therefore, of the rosettes arising by consecutive divisions of this kind. In these rosettes the whips are directed outward, in marked contrast to the culture rosettes of *Tr. Lewisi*. This fact, it may be well to note, corresponds to Schaudinn's observation on the life-cycle of the "leucocytozoon," in which case the trypanosomes which develop in the gut of the mosquito often agglutinate with their whips directed outward.

THE TOXIN OF TR. BRUCEI.

Kanthack, Durham, and Blandford[9] showed that the blood serum from which the abundant trypanosomes had been removed by filtration had no specific toxic effect, and that enormous doses of blood swarming with the protozoa produced no immediate symptoms. Rich Nagana blood kept for several days until the protozoa had disappeared from it was likewise without toxic effect. From these and other similar experiments they regard the blood as free from specific poison. The successful cultivation of this organism opened up a new way in which to attack this problem. Cultures

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which have developed at 25° C. may be readily deprived of living trypanosomes by exposure for five days to a temperature of 34° C. The whole condensation fluid from four flasks ($\frac{3}{4}$ c.c.) treated in this manner was injected into the peritoneal cavity of a white mouse weighing 22 g., without any apparent ill effect. Another mouse of about the same size was injected with $\frac{1}{3}$ c.c. of the condensation fluid from four flasks grown at 25° for fifteen days, and then exposed to 34° for two days. This material still contained actively motile trypanosomes. The animal survived, and when inoculated three weeks later with virulent Nagana blood died in six days. These experiments indicate that *Tr. Brucei* produces no powerful stable toxin.

In the course of some attempts to immunize guinea pigs to this disease we have obtained some evidence of the probable presence of a toxin. The first two or three subcutaneous injections of attenuated cultures have little local effect. A rise in temperature and a loss in weight are observed. After several protective inoculations the quickness of reaction on the part of the animal is increased. The temperature rises within a few hours after the injection, the animal shows signs of pain at the point of inoculation, and often lies down. After a few days a hard eschar forms at this point. This dries and sloughs off after a week or two, and the resulting ulcer heals with scar formation, after which the animal entirely recovers. Subsequent injections produce similar effects. The more or less complete general immunity is then an important factor in this intense local poisoning, and seems to indicate that the toxin becomes active upon the destruction of the trypanosome cells in the body of the living host.

CONJUGATION.

In a recent publication Prowazek[22] announces in a few lines the observation of conjugation in *Tr. Brucei* without giving any definite description of the process. Reviewing the question of a sexual process among flagellates in a later publication, he[23] points out the lack of information on this point. Regarding as conjugation only those fertilization processes in which two cells take part, the positive cases are few indeed.

Schaudinn[27] describes macrogamete and microgamete forms of

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the halteridium, *Hemoproteus noctuæ*, which he reclassifies as a trypanosoma, and the union of these forms to produce an oökinete. Whether this protozoon is properly classified as a trypanosome may well be questioned. At any rate, nothing corresponding to his macrogametes and microgametes or to their precursors, the gametocytes, has ever been described in the cycles of such well-known and representative forms as *Tr. Lewisi* and *Tr. Brucei.*

The conjugation described by Plimmer and Bradford has been regarded as erroneous by the continental workers, and the idea that the smaller chromatin body is merely a base for the flagellum seems to be the most widely accepted view at the present time. However, the observations of Plimmer and Bradford of changes in this body during the so-called agglutination of the trypanosomes are readily confirmed. Stained preparations made of the tail blood of a mouse in a dying condition were found to be particularly rich in these couples. The contact is not merely at the extreme tips, but there is an overlapping of the two ends, and very often such intimate contact that most careful examination at a magnification of three thousand diameters fails to find any line of separation. There appears to be complete fusion and continuity of protoplasm between the cells. One naturally looks carefully for signs of unusual activity on the part of the smaller chromatin body under these circumstances. These bodies frequently lie very close together in these couples, sometimes in such intimate relation that they appear as one body under the greatest magnification. In other cases the two bodies lie close to each other and appear to be connected by a lighter band, as in Plate XVI, Fig. 3. Again a tetrad of four blepharoplasts may be found at the junction, and it is impossible to determine to which cell they may belong (Plate XVI, Fig. 4). Such double trypanosomes are so numerous in the blood of the dying mouse that often three or four of them are to be found in every field of the microscope. They would seem to indicate an actual conjugation effected by changes in the blepharoplast or micronucleus, as Plimmer and Bradford call it. It is impossible to be certain of such a process merely from study of stained preparations, however. The question appears worthy of further study.

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In the rat trypanosome the blepharoplast usually has a bilobed appearance in stains made from blood containing agglutinations. Rarely actual fragmentation of this body into equal portions is seen. Exchange of material from one cell to another has not been demonstrated.

CONCLUSIONS.

1. The division of trypanosomes is not exactly longitudinal, but always more or less oblique in direction. The whip does not divide, but a new one is formed in each division. There is only one type of division in the mammalian host and in cultures on blood agar. The process has been observed throughout under the microscope in the case of *Tr. Lewisi* and *Tr. Brucei*.

2. The destruction of trypanosomes in the blood is due to a cytolytic action of the blood plasma rather than to actual phagocytosis. The solution of *Tr. Brucei* in the later stages of Nagana probably sets free the toxin, aud thus produces the characteristic symptoms and death. This accounts for the remarkable fact that in some cases the blood may be swarming with the protozoa without marked symptoms on the part of the host, and again another animal may die from the effects of relatively few trypanosomes in the blood. Human trypanosomiasis is an example of the latter class.

3. The culture rosettes of *Tr. Lewisi* have their flagella directed centrally, while those of *Tr. Brucei* are more irregular, but as a rule the whips are on the periphery.

4. The agglutination of trypanosomes is often associated with changes in the smaller chromatin body, suggesting an isogamic conjugation.

This work has been supported by the grant of a Fellowship in the Rockefeller Institute for Medical Research. It has enjoyed the constant direction and tireless supervision of Professor Novy, who has been the mind and spirit of the investigations. I appreciate it a high honor to be allowed to express my indebtedness to him. I am also indebted to Dr. J. F. Eastwood for assistance in the preparation of the photomicrographs, and to Professor Huber for many valuable suggestions.

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EXPLANATION OF PLATES XI-XVII.

PLATE XI.—DIVISION OF TR. LEWISI IN CULTURE.

FIG. 1.—Culture rosette; living preparation on Ranvier slide; at 8:25 A. M.

FIG. 2.—Same at 8:30 A. M. Two cells are in process of division.

FIG. 3.—8:35 A. M.: Cell *a* has divided, *d* is beginning to shorten.

FIG. 4.—8:38 A. M.: Cell *d* is shortening rapidly.

FIG. 5.—Sketched at 8:41 A. M.

FIG. 6.—8:44 A. M.: The daughter-cells of *a* have elongated and become actively motile. The flagella are not definitely seen.

FIG. 7.—8:47 A. M.: Cell *e* is shortening.

FIG. 8.—8:50 A. M.: Cell *b* has become motionless and evidently dead.

FIG. 9.—8:55 A. M.: Three of the cells are in process of division.

FIG. 10.—9:10 A. M.: The increasing motility of the lengthening cells has altered the relative positions of the cells.

FIG. 11.—9:28 A. M.: The motility of the rosette is increasing. Cell *b* continues motionless.

FIG. 12.—9:39 A. M.: All the daughter-cells have elongated and become active, measuring now about 10 μ in length.

Magnified about 1,400 diameters.

PLATE XII.—DIVISION OF TR. BRUCEI IN CULTURE.

FIG. 1.—10:30 A. M.: A large cell fixed by its posterior tip.

FIG. 2.—8:55 P. M.: A second whip has formed.

FIG. 3.—Next day 8:52 A. M.: The new whip is larger and more active. Division of the protoplasm is apparent on focusing.

FIG. 4.— 10 A. M.: Complete division can be made out by focusing.

Magnified about 2,000 diameters.

PLATE XIII.—TR. LEWISI.

The microphotographs were all taken, with exception of Fig. 3, Plate XIV, at a magnification of 3,000 diameters, and the reproductions are about three-fourths of the original size.

FIG. 1.—*Tr. Lewisi*, adult stage; blood of rat fifteen days after inoculation.

FIG. 2.—Anomalous form frequently found during the developmental stage; in blood of a rat, six days after inoculation..

FIG. 3.—Cell enlarged preparatory to division; a new whip beginning to grow out from its base near the blepharoplast, the latter not yet divided.

FIG. 4.—Later stage, two distinct whips, blepharoplasts and nuclei, two more whips forming from the elongated blepharoplasts.

PLATE XIV.—TR. LEWISI.

FIG. 1.—Four-cell stage, each cell in process of further division. This specimen has been crushed in spreading so as to destroy the outlines of the cells, a common occurrence.

FIG. 2.—Eight-cell rosette. Note the long original or parent whip on one of the cells. Several of them show a second whip growing out preparatory to a further division.

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PLATE XI.

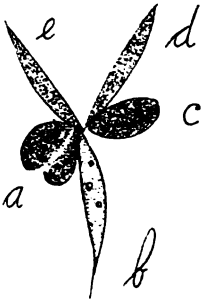
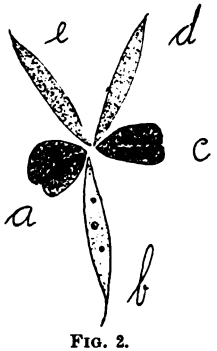


FIG. 1.



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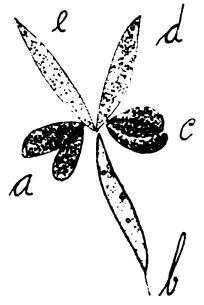
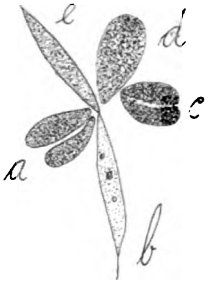


FIG. 3.



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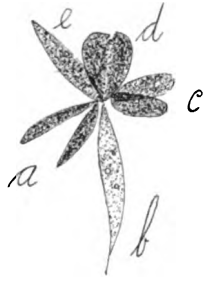

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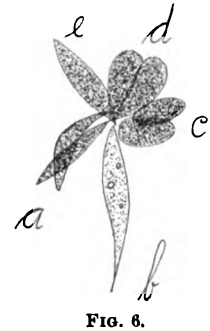

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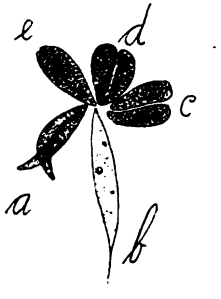

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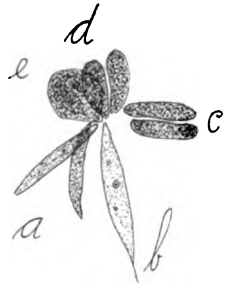

FIG. 8.

FIG. 9.

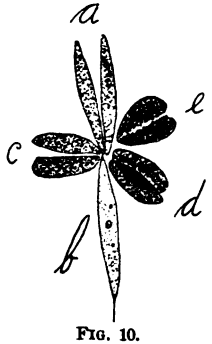

FIG. 10.

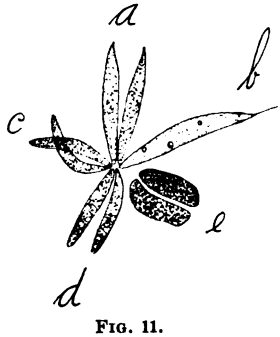

FIG. 11.

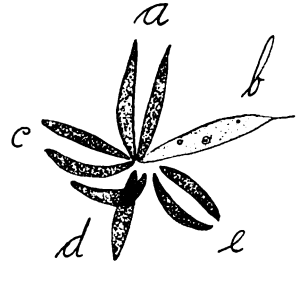

FIG. 12.

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PLATE XII.

FIG. 1.

FIG. 2.

FIG. 3.

FIG. 4.

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PLATE XIII.

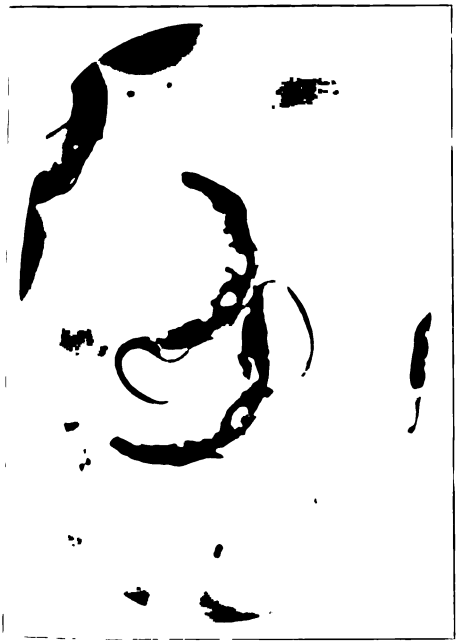

FIG. 1.

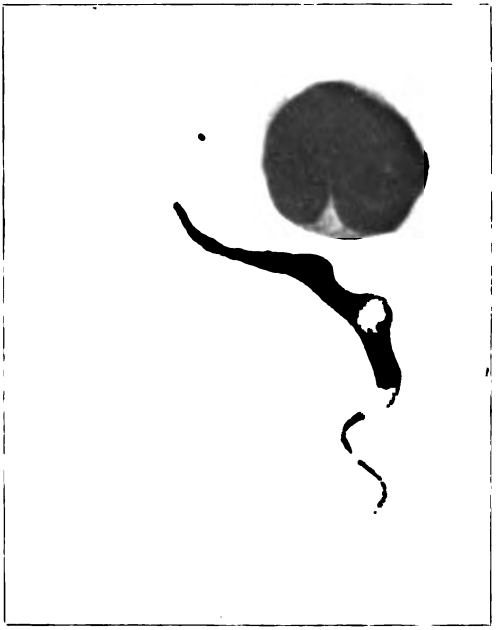

FIG. 2.

FIG. 3.

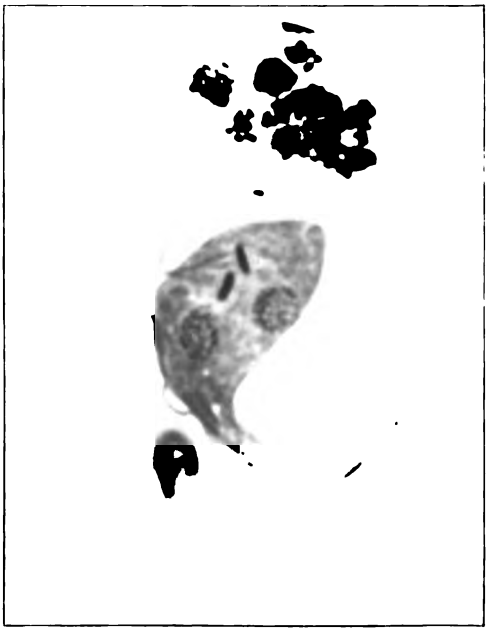

FIG. 4.

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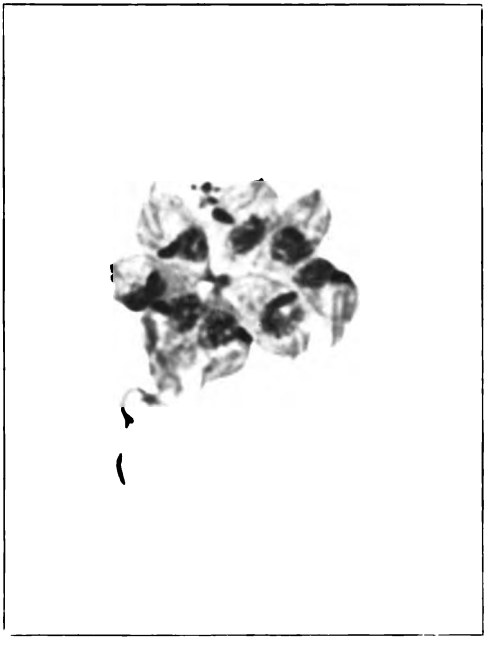
PLATE XIV.

FIG. 1.



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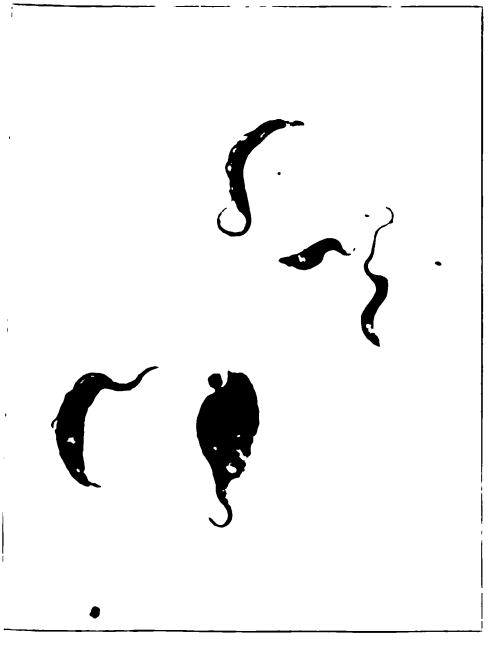

FIG. 3.

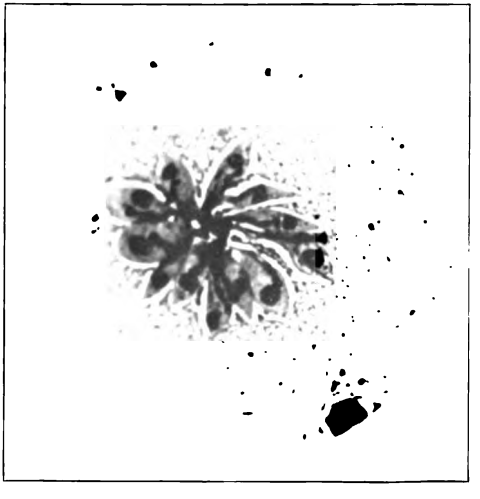

FIG. 4.

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PLATE XV.

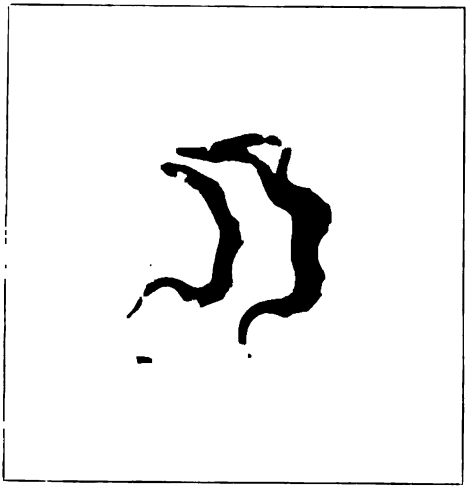

FIG. 1.

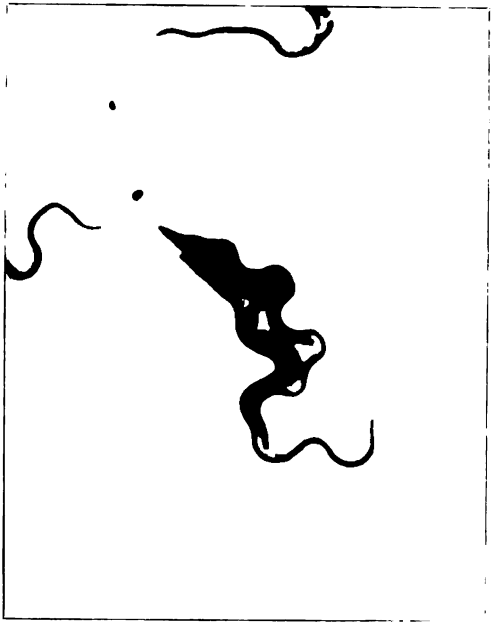

FIG. 2.

FIG. 3.

FIG. 4.

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PLATE XVI.

FIG. 1.

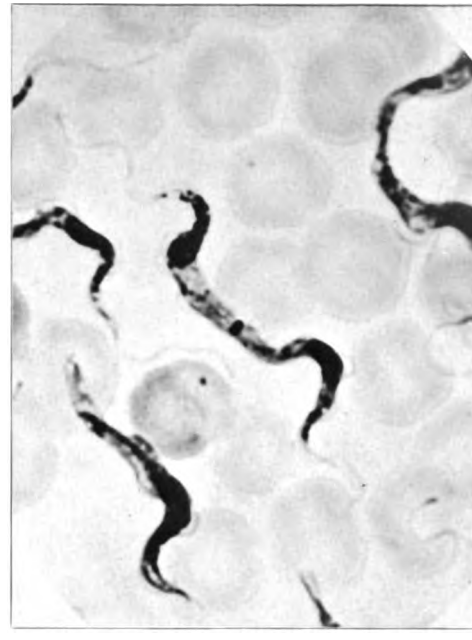

FIG. 2.

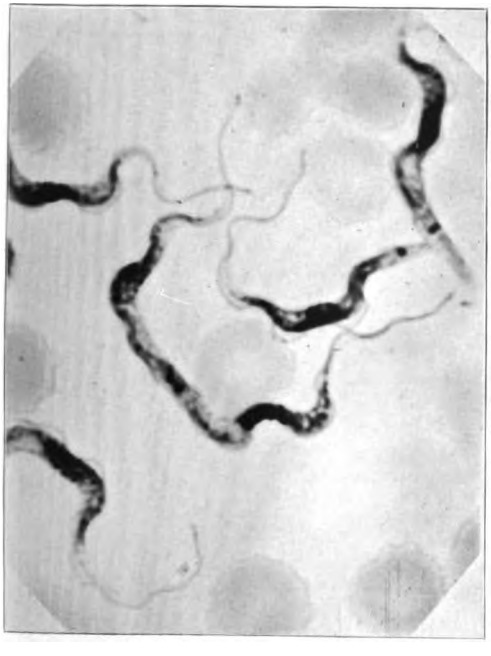

FIG. 3.

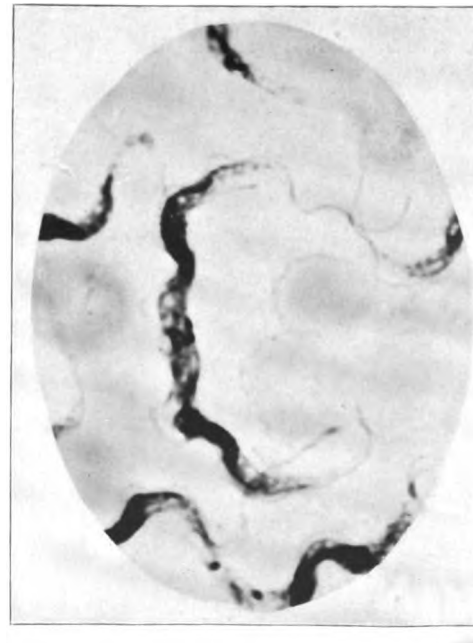

FIG. 4.

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PLATE XVII.

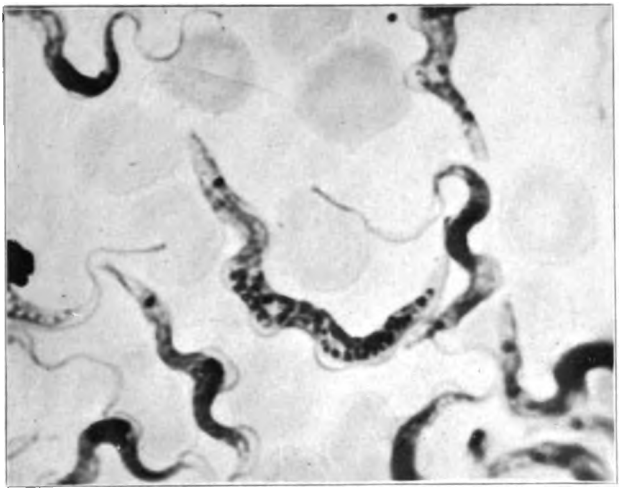

FIG. 1.

FIG. 2.

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FIG. 3.—Different stages in the growth of the small to the large, dividing form, in rat blood six days after inoculation. ×1,500, but reduced three-fourths.

FIG. 4.—Rosette of *Tr. Lewisi* in culture on blood agar. Note the central direction of the flagella.

PLATE XV.—TR. BRUCEI.

FIG. 1.—Large and small form. The small cell is the product of a recent division; the large one is preparing to divide.

FIG. 2.—Division into two. Note the projection of one cell posterior to the other. A third whip is forming.

FIG. 3.—Later stage, one cell about to separate.

FIG. 4. –Early stage of division showing independent growth of the new whip. The undulating membrane has been dissolved away by alkaline phosphate solution.

PLATE XVI.—TR. BRUCEI.

FIG. 1.—Agglutination of two similar trypansomes.

FIG. 2.—Two cells in intimate contact with only one blepharoplast to be made out. Conjugation?

FIG. 3.—Another couple showing the blepharoplasts apparently connected by a chromatin band.

FIG. 4.—A couple with four of the smaller chromatin bodies.

PLATE XVII.—TR. BRUCEI.

FIG. 1.—One kind of the granular form. The nucleus is completely fragmented. Note the long body and relatively short flagellum.

FIG. 2.—Large hyaline trypanosome much less deeply stained than the ordinary type. The nucleus is round.

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Separatabdruck aus dem „Zentralblatt für Physiologie"
5. November 1904. — Bd. XVIII. Nr. 16.

From the Physiological Laboratory of Columbia University, New York.[1])

Ein Beitrag zur Viskosität des Blutes.

Von **R. Burton-Opitz.**

(Der Redaktion zugegangen am 26. September 1904.)

Im Laufe des letzten Winters hatte ich Gelegenheit, die Viskosität des Blutes zweier Hunde zu bestimmen, deren Schilddrüsen bedeutend vergrößert waren. Die Versuche wurden nach der von Hürthle[2]) angegebenen Methode ausgeführt.

Die rechte Drüse des einen Tieres wog 57, die linke 50 g. Sie besaßen jede eine Länge von 80, eine Breite von 40 und eine Höhe von 25 mm. Die Drüsen des zweiten Hundes wogen 29 und 26 g. Im Einklange mit ihrer Schwere waren sämtliche zu- und abführenden Blutgefäße sehr stark erweitert. Die Karotiden sowie die entsprechenden Jugularvenen waren mindestens zweimal so stark als sie bei Hunden dieser Größe eigentlich hätten sein sollen.

Hund I wurde zwei Wochen im Institut gehalten, ehe die Viskositätsbestimmungen ausgeführt wurden. Während dieser Zeit nahm er das Futter regelmäßig und zeigte außer allgemeinen, oft sehr lebhaften Muskelzuckungen, keine anderen auf eine Affektion der Schilddrüse hinweisenden Symptome. Hund II dagegen wurde schon am ersten Tage nach seiner Einlieferung von krampfartigen Anfällen ergriffen, die während der folgenden Tage bedeutend stärker und öfter auftraten. Da die Temperatur des Tieres am vierten Tage auf 39·0° C stieg, wurde es noch an demselben Abende für den Versuch benutzt. Das letzte Futter hatte es 20 Stunden vorher erhalten.

Die Versuche wurden mit einer Glaskapillare ausgeführt, deren Länge 245·5 mm und deren Durchmesser 0·6636 mm betrugen. Die Tiere erhielten Morphin und wurden sodann mit einer Mischung von Chloroform und Aether narkotisiert.

Die Ergebnisse der Versuche sind in der folgenden Tabelle zusammengefaßt. Die ersten sieben Bestimmungen betreffen Hund I, die letzten vier Hund II.

[1]) Conducted under the auspices of the Rockefeller Institute for medical research.

[2]) C. Hürthle. Pflügers Archiv LXXXII (1900), S. 415 bis 442.

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Nummer der Versuche		Spezif. Gewicht d. Blutes	Temperatur d. Tieres	Ausgeflossene Menge mg.	Zeit Sek.	Druck mm Hg.	Koeffizient K.	Größen-Differenz	Mittel von K.
Hund I 12 Kilo	1	1·05028	37·0	2209·9	15·54	134·0	1279·30	78·58	1232·67
	2	1·05028	37·0	1547·7	11·40	134·2	1219·50		
	3	1·05028	37·0	1360·8	10·86	125·8	1200·72		
	4	1·05028	37·0	1513·6	11·16	133·1	1228·30		
	5	1·05028	37·0	1680·5	11·34	145·2	1230·20		
	6	1·05028	37·0	1611·0	11·30	140·0	1227·50		
	7	1·05028	37·0	1727·0	12·35	135·6	1243·20		
Hund II 8 Kilo	1	1·04695	39·1	1892·5	11·10	163·5	1261·08	115·72	1257·3
	2	1·04695	39·1	1699·8	10·55	159·4	1222·35		
	3	1·04695	39·1	1946·9	11·29	156·7	1330·82		
	4	1·04695	37·8	1627·0	10·66	151·9	1215·10		

Diese Uebersicht zeigt, daß die Viskosität des Blutes dieser Tiere ungemein gering ist. Die Koeffizienten besitzen die Werte 1232 und 1257. Vergleicht man diese mit dem für destilliertes Wasser von 37° C angegebenen Koeffizienten (Poiseuille), so findet man, daß die Viskosität des Blutes dieser Hunde sich zu der des destillierten Wassers wie 3·7 : 1 verhält.

An normalen Hunden und Kaninchen ausgeführte Versuche[1]) haben ergeben, daß die Viskosität des Blutes dieser Tiere die Mittelwerte 925 (5·0 : 1), resp. 1346 (3·4 : 1) besitzt. Die oben angegebenen Werte sind daher beinahe so niedrig, wie die für das Blut des Kaninchens gefundenen. Auch sind sie bedeutend niedriger wie die Werte, welche für das Blut zweier hungernder Hunde in der eben zitierten Arbeit angeführt sind. K betrug in den letzten beiden Fällen 1110 und 1101.

In Uebereinstimmung mit der geringen Viskosität betrug das spezifische Gewicht des Blutes des ersten Hundes nur 1·050 und das des zweiten nur 1·046.

Die Gerinnungszeit des Blutes betrug bei Hund I 28 und bei Hund II 20 Minuten. Es ist mir wiederholt aufgefallen, daß die Viskosität und die Gerinnungszeit des Blutes ein umgekehrtes Verhältnis zueinander bewahren, d. h. je geringer die Viskosität, desto länger ist die Gerinnungszeit.

In einer weiteren, noch nicht abgeschlossenen Reihe von Versuchen wurde der Einfluß geprüft, welchen intravenöse Injektionen von Schilddrüsenextrakt auf die Viskosität des Blutes ausüben. Die Injektionen bewirkten immer eine Erhöhung der Viskosität. Die totale Exstirpation der Schilddrüsen war jedoch nicht immer von einer Erniedrigung der Viskosität gefolgt. Die Frage, ob dieses teilweise negative Resultat auf die Anwesenheit kleinerer Nebendrüsen zurückzuführen sei, soll durch weitere Versuche geprüft werden.

[1]) R. Burton-Opitz. Pflügers Archiv LXXXII (1900), S. 447 bis 463.

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[*Reprinted from the Journal of Physiology.*
Vol. XXXII. No. 1, *December* 30, 1904.]

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THE CHANGES IN THE VISCOSITY OF THE BLOOD PRODUCED BY ALCOHOL[1]. By R. BURTON-OPITZ.

(*From the Physiological Laboratory of Columbia University, at the College of Physicians and Surgeons, New York.*)

THE viscosity-values forming the basis of this paper have been obtained by the method described by Hürthle[2]. Briefly outlined it consists in determining the time which a measured quantity of blood, under a certain pressure, requires to pass through a capillary tube of known size. Cannulas are placed in both carotid arteries. The cannula in the left carotid is connected with a mercury-manometer, while the one in the right is connected with one end of the capillary tube. When the blood escapes from the other end of the capillary tube, it is caught upon one of two triangular glass plates which are attached to a vertical lever and move from side to side in front of the lumen of the tube. The glass plates diverge below and lead to two small receptacles. After a few drops of blood have escaped into one of the small glasses standing below the plates, the lever is rapidly moved to the other side. The blood is now allowed to flow into the second weighing glass during a period of from 15 to 30 seconds, when it is again diverted into the receptacle used previously. The movements of the vertical lever are recorded upon a rapidly moving drum beneath the record of a Jaquet chronograph marking fifths of seconds. By this means the length of the period is determined during which the blood is allowed to flow into the weighing glass.

The viscosity coefficient K is obtained subsequently by calculation. For this purpose it is essential to know the quantity of blood collected during a definite period, its specific gravity, the blood-pressure and the length and internal diameter of the capillary. In order that these values may become more intelligible to the reader, they are compared

[1] This study has been conducted under a grant from the Rockefeller Institute for Medical Research.

[2] Hürthle, K., *Archiv für die ges. Physiol.*, LXXXII. pp. 415—442. 1900.

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with K for distilled water at 37° C., which, according to Poiseuille, is 4700. Thus, the viscosity of the blood is denoted in this investigation in terms of multiples of K for distilled water at 37° C.

In the present series of experiments only one capillary tube was employed. By using the same tube for every determination, it was hoped to render wide variations between the different coefficients practically impossible and even insignificant changes in the viscosity more easily perceptible. The cleaning and readjusting of the capillary tube made the intervals between the different determinations necessarily long; however, as the viscosity remains constant for a long time, no errors were introduced by the lengthening of this period. After the injections of alcohol, the experiments were made, as a rule, at intervals of from 10 to 15 minutes.

The capillary measured 245·5 mm. in length ($\log l = 2{\cdot}39005$), and its diameter, determined by means of the micrometer scale, 0·6636 mm. ($\log d^4 = 0{\cdot}28760 - 3$). The accuracy of this capillary tube was tested first by a number of experiments with distilled water. Having given coefficients which closely corresponded with those found by Poiseuille, the capillary used was considered reliable for the experiments in hand.

The Changes in the Viscosity of the Blood produced by Intravenous Injections of Small Quantities of Distilled Water and 0·7 per cent. NaCl Solution.

As the alcohol was to be introduced in solution with distilled water or 0·7 per cent. NaCl, it became necessary to determine, first of all, whether these fluids produce material changes in the viscosity when used alone. As relatively small quantities of the alcoholic solution were injected, correspondingly small amounts of the diluting fluids were experimented with.

No special feeding of the dogs used in these experiments was necessary, the normal viscosity having no bearing upon the question at issue. A mixed diet, consisting of meat and carbohydrates, was given to them until the day of the experiment. After the administration of small amounts of morphine, the animals were kept in a uniformly light chloroform-ether narcosis. It has been shown previously[1] that the viscosity is not changed by these narcotics, when administered in moderate doses.

[1] Burton-Opitz, R., *Archiv für die ges. Physiol.*, LXXXII. pp. 448—450. 1900.

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The method followed in each experiment of this series was as follows. Two or more determinations were made first to obtain the normal viscosity coefficient of the blood. A measured quantity of NaCl solution or distilled water was then allowed to flow into the facial vein, the injection being made very slowly in every case. After an interval of from 5 to 10 minutes, the viscosity was again measured by two or more determinations.

The details of the experiments with distilled water and NaCl solution are given in Tables 1 and 2. Experiments 1, 2, 3 and 4 deal with the changes resulting from the injection of distilled water, and Experiments 5, 6, 7 and 8 show the changes produced by the 0·7 sodium chloride solution. Each experiment is subdivided into two parts, showing, on the one hand, the normal viscosity of the blood, and, on the other, the viscosity subsequent to the injection. Among other details the tables also contain the quantity of fluid injected, the length of the period intervening between the introduction of the fluid and the beginning of each determination, the rectal temperature of the animal and the specific gravity of the blood before and after the injection. The latter was obtained, as a rule, immediately after the completion of each set of viscosity determinations. Small pycnometers were used for this purpose. In the last column of each table K for the blood is compared with K for distilled water at 37° C.

These experiments prove conclusively that the fluids used in diluting the alcohol produce, when injected intravenously, a slight, but nevertheless constant change in the viscosity of the blood. It should be noted, however, that these fluids exhibit, at least at first, directly antagonistic tendencies. The 0·7 per cent. NaCl solution causes an immediate and very distinct decrease in the viscosity, while equal amounts of distilled water render the blood slightly more viscous.

Considering the relative insignificance of these changes, the effect of the diluting fluids may be disregarded completely, if the intravenous injection of the fluids plus the alcohol produces results of a more decisive character.

The Changes in the Viscosity of the Blood produced by Injections of Alcohol.

The method followed in the experiments with alcohol is the same as that outlined in a preceding paragraph. A number of determinations

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TABLE I. *The Viscosity of the Blood after Intravenous Injection of Distilled Water.*

Number of exp. and weight of dog	Determination number	Time elapsed since injection, minutes	Temperature of animal, °C.	Specific gravity of the blood	Quantity escaped from cap., mg.	Time, seconds	Pressure, mm. H_g	Viscosity coefficient K	Difference between K of same group	Mean value of K	Increase of viscosity equals	Relation viscosity coefficient to that dist. H_2O at 37° C.
Exp. 1 16 kg. Normal	1	—	37·1	1·0521	1786·3	16·00	132·50	1018·90	16·95	1005·42	47·79	4·67
Exp. 1 16 kg. After inj. of 10 c.c.	2	—	,,		2305·5	20·02	139·00	996·95				
	3	5	,,	1·0527	1783·8	15·32	144·40	969·75	24·28	957·63		4·90
	4	12	36·8		1591·1	16·19	125·00	945·52				
Exp. 2 19 kg. Normal	1	—	37·0	1·0568	1573·2	19·57	119·90	803·2	1·1	802·7	16·7	5·85
Exp. 2 19 kg. After inj. of 10 c.c.	2	—	,,		2119·2	22·21	142·50	802·1				
	3	10	,,	1·0589	1897·3	21·92	133·50	775·2	21·7	786·0		5·98
	4	20	36·2		1888·1	21·59	131·20	796·9				
Exp. 3 7 kg. Normal	1	—	36·9	1·0561	1716·1	15·78	130·60	998·30	10·49	993·05	24·60	4·78
Exp. 3 7 kg. After inj. of 20 c.c.	2	—	,,		1239·9	11·47	131·20	987·81				
	3	10	,,	1·0565	2065·9	20·83	122·20	972·62	8·34	968·45		4·85
	4	16	,,		2033·0	19·57	129·10	964·28				
Exp. 4 13 kg. Normal	1	—	36·4	1·05988	1563·7	12·40	170·20	885·11	8·10	889·61	23·49	5·28
Exp. 4 13 kg. After inj. of 20 c.c.	2	—	,,		1393·2	11·63	157·50	893·21				
	3	10	,,	1·06070	1390·2	10·95	177·10	855·68	20·88	866·12		5·42
	4	15	,,		1615·8	13·12	167·70	876·56				

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TABLE II. *The Viscosity of the Blood after Intravenous Injection of 0·7 per cent. NaCl Solution.*

Number of exp. and weight of dog		Determination number	Time elapsed since injection, minutes	Temperature of animal, °C.	Specific gravity of the blood	Quantity escaped from cap., mg.	Time, seconds	Pressure, mm. Hg	Viscosity coefficient K	Differences between K of same group	Mean value of K	Decrease of viscosity equals	Relation viscosity coefficient to that dist. H_2O at 37° C.
Exp. 5	Normal	1	—	37·0	1·06180	858·3	11·54	123·00	721·05	1·13	721·61	19·31	6·51
		2	—	,,		1124·2	14·17	131·00	722·18				
	After inj.	3	5	,,	1·06175	1211·2	13·97	138·30	747·50	13·15	740·93		6·34
8 kg.	of 5 c.c.	4	10	,,		1098·0	15·02	118·70	734·35				
Exp. 6	Normal	1	—	36·8	1·06105	1203·1	10·98	165·20	791·43	2·60	790·13	48·89	5·94
		2	—	,,		1646·9	14·40	173·00	788·83				
	After inj.	3	10	,,	1·06015	1625·6	13·15	174·20	847·51	16·97	839·02		5·60
12 kg.	of 30 c.c.	4	15	36·0		1420·6	11·74	174·00	830·54				
Exp. 7	Normal	1	—	36·8	1·06188	1580·2	13·47	170·10	822·30	23·53	834·06	26·08	5·63
		2	—	,,		1471·3	13·93	149·00	845·83				
	After inj.	3	5	,,	1·06108	1423·7	13·29	147·10	868·95	17·61	860·14		5·46
11 kg.	of 20 c.c.	4	12	36·4		1778·6	13·88	179·60	851·34				
Exp. 8	Normal	1	—	36·5	1·05860	1162·7	12·06	125·40	919·86	33·03	906·94	110·24	5·18
		2	—	,,		1634·4	17·50	126·00	886·83				
		3	—	,,		1711·7	12·65	177·10	914·14				
	After inj.	4	10	,,	1·05320	2081·4	15·24	161·50	1016·58	1·20	1017·18		4·62
11 kg.	of 50 c.c.	5	15	,,		1852·8	12·79	171·10	1017·78				

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were made before, as well as after, the injection. The alcohol, diluted with a 0·7 per cent. NaCl solution or distilled water, was introduced first into the facial vein and, in another set of experiments, into the stomach or duodenum. The alcoholic solution was used in strengths varying from 10 to 60 per cent.

Those animals, in which the alcohol was introduced directly into the intestinal canal, received no food on the day of the experiment. A small incision was made in the median line, and the pylorus drawn forward and occluded by a ligature. The alcohol was then injected either into the stomach or the duodenum, and the wound again closed.

It was proved by two preliminary experiments that the viscosity of the blood is not changed by this operative procedure.

The experiments are recorded in full in Tables 3, 4, and 5. Table 3 contains the results of three experiments in which the alcohol was introduced into the facial vein; Table 4, three experiments in which the alcohol was injected into the stomach; and Table 5, two experiments in which the injection was made into the duodenum. In the second experiment of each group a 0·7 per cent. saline solution was used for dilution, while in the others distilled water was employed.

Whether introduced directly into the circulation, or into the digestive tract, the alcoholic solutions employed in these experiments always *rendered the blood more viscous.* By far the greatest increase appeared when the alcohol was injected into the stomach or the duodenum. Thus we observe[1], for example, that after injecting 30 c.c. of a 60 per cent. solution into the stomach, the viscosity coefficient changed from 948·94 to 822·59. This implies that the viscosity which normally was only 4·95 times as great as that of distilled water at 37° C. became 5·71 times as great. However, the other experiments of this series gave equally decisive results.

The increase produced by intravenous injections of alcohol was never so pronounced. This may be due in part to the fact that relatively small quantities of weak alcoholic solutions had to be used for obvious reasons. As the NaCl solution alone decreases the viscosity, the NaCl solution plus the alcohol had to overcome this effect first before it could produce a decided change in the opposite direction. We have also seen previously that small quantities of distilled water rendered the blood slightly more viscous. The same change is produced by distilled water plus alcohol; but in the latter case the increase in the viscosity was

[1] See Exp. 2, Table IV.

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TABLE III. *The Viscosity of the Blood after Intravenous Injections of Alcohol.*

Number of exp. and weight of dog.		Determination number	Alcohol c.c.	Alcohol per cent.	Time elapsed since injection, minutes	Temperature of animal, °C.	Specific gravity of the blood	Quantity, mg.	Time, seconds	Pressure, mm. H_g	Viscosity coefficient K	Difference between K of same group	Mean value of K	Increase of viscosity equals	Relation viscosity coefficient to that dist. H_2O at 37° C.
Exp. 1 14 kg.	Normal	1	—	—	—	36·5	1·0564	1159·4	18·51	103·50	953·42	10·64	948·10	26·67	4·95
		2	—	—	—	,,		1266·3	15·39	104·60	942·78				
	After inj.	3	5	10	2	,,	1·0572	1250·2	14·28	112·90	928·65	35·40	921·43		5·10
		4	—	—	10	,,		1151·1	13·81	106·70	935·52				
		5	—	—	15	,,		1487·0	16·15	122·50	900·12				
Exp. 2 9 kg.	Normal	1	—	—	—	36·5	1·0638	1885·0	15·38	150·60	710·78	16·08	718·74	21·10	6·54
		2	—	—	—	,,		1843·7	14·67	150·00	726·76				
	After inj.	3	3	25	1	,,	1·0649	1848·4	16·52	137·80	704·21	18·14	697·64		6·73
		4	—	—	11	,,		1690·3	20·58	141·80	691·07				
Exp. 3 8 kg.	Normal	1	—	—	—	37·0	1·06180	1098·0	15·02	118·70	734·35	18·15	740·92	37·61	6·34
		2	—	—	—	,,		1211·2	13·97	138·80	747·50				
	After inj.	3	5	25	2	,,	1·06240	1174·8	14·85	152·50	711·55	16·48	703·31		6·68
		4	—	—	10	,,		971·2	13·30	125·20	695·07				

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TABLE IV. *The Viscosity of the Blood after Injections of Alcohol into the Stomach.*

Number of exp. and weight of dog		Determination number	Alcohol inj. c.c.	Alcohol inj. per cent. sol.	Time elapsed since injection, minutes	Temperature of animal, °C.	Specific gravity of the blood	Quantity, mg.	Time, seconds	Pressure, mm. Hg.	Viscosity coefficient K	Difference between K of same group	Mean value of K	Increase of viscosity equals	Relation viscosity coefficient to that dist. H_2O at 17° C.
Exp. 1 11 kg.	Normal	1	—	—	—	37·0	1·0662	2133·3	23·04	157·70	697·06	56·02	664·16	56·07	7·07
		2	—	—	—	,,		1669·2	21·03	144·00	654·40				
		3	—	—	—	,,		1311·3	15·76	154·10	641·04				
	After inj.	4	30	25	10	,,	1·0673	1327·9	17·79	140·50	630·24	43·50	608·09		7·72
		5	—	—	20	,,		1761·0	22·34	153·98	607·29				
		6	—	—	30	36·1		1145·7	17·22	134·52	586·74				
Exp. 2 10 kg.	Normal	1	—	—	—	36·9	1·05921	1130·5	10·46	142·30	907·85	77·69	948·94	126·35	4·95
		2	—	—	—	,,		1104·7	9·91	135·20	985·54				
		3	—	—	—	,,		981·3	9·14	134·60	953·44				
	After inj.	4	30	60	10	,,	1·06040	665·2	9·71	97·30	840·66	39·73	822·59		5·71
		5	—	—	20	,,		873·2	11·06	114·10	826·18				
		6	—	—	30	,,		986·0	10·69	137·50	800·93				
Exp. 3 11 kg.	Normal	1	—	—	—	36·7	1·05675	1297·5	11·68	134·80	987·33	53·02	1006·58	99·64	4·66
		2	—	—	—	,,		1481·0	12·99	131·30	1040·35				
		3	—	—	—	,,		1246·0	11·71	128·50	992·07				
	After inj.	4	30	50	15	,,	1·05820	1162·7	12·06	125·40	919·86	33·03	906·94		5·18
		5	—	—	30	,,		1634·4	17·50	126·00	886·83				
		6	—	—	45	,,		1711·7	12·65	177·10	914·14				

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TABLE V. *The Viscosity of the Blood after Injections of Alcohol into the Duodenum.*

Number of exp. and weight of dog		Determination number	Alcohol inj. c.c.	Alcohol inj. per cent. sol.	Time elapsed since injection, minutes	Temperature of animal, ° C.	Specific gravity of the blood	Quantity, mg.	Time, seconds	Pressure, mm. Hg	Viscosity coefficient K	Difference between K of same group	Mean value of K	Increase of viscosity equals	Relation viscosity coefficient to that dist. H_2O at 37° C.
Exp. 1 10 kg.	Normal	1	—	—	—	37·0	1·0585	1259·8	11·90	133·90	945·70	30·51	928·05	120·41	5·06
		2	—	—	—	,,		1468·5	14·68	129·60	923·26				
		3	—	—	5	,,		1869·6	14·32	125·00	915·19				
	After inj.	4	40	25	15	,,	1·0593	1100·4	15·09	107·70	809·25	48·57	807·64		5·61
		5	—	—	30	,,		1276·4	16·41	118·70	782·55				
		6	—	—		,,		1668·9	20·10	119·40	831·12				
Exp. 2 18 kg.	Normal	1	—	—	—	36·6	1·06070	1615·8	13·12	167·70	876·56	20·88	866·08	113·38	5·42
		2	—	—	—	,,		1390·2	10·95	177·10	855·68				
	After inj.	3	30	50	15	,,	1·06158	1662·1	15·95	158·60	783·51	61·61	752·70		6·24
		4	—	—	30	,,		1597·2	17·59	150·00	721·90				

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somewhat greater than when distilled water alone was used. These facts eliminate the possibility that the greater viscosity of the blood after intravenous injections of alcohol plus distilled water is due solely to the diluting fluid.

When the time elapsed since the injection is taken into consideration, it is seen that the alcohol produces very quick effects even when introduced into the stomach or the duodenum. In the latter experiments an almost maximal increase in the viscosity appeared in from five to ten minutes after the injection. Moreover, the effect is lasting, the coefficient showing practically no change during the succeeding thirty to forty-five minutes. The specific gravity was determined in two experiments an hour and a half after the injection. Even at this time it did not show a material decrease in its value, indicating thereby that the viscosity continued as yet at its former level.

The latter deduction is perfectly permissible, because it has been demonstrated[1] that the specific gravity of the blood may be regarded as a safe index of the viscosity. The greater the specific gravity, the greater the viscosity, and *vice versâ*. No exception to this rule was met with in the experiments just related.

The coefficients for the normal blood of the dog obtained from the present sixteen experiments vary between 1006 and 664, average 859. The viscosity of the blood is therefore 5·5 times as great as that of distilled water at 37° C. In earlier experiments of this kind[2] *K* for the blood of the dog was found to be 925. Considering that no attempt has been made at this time to vary the viscosity of the blood by feeding, the results may be said to correspond very closely with one another.

[1] Burton-Opitz, *loc. cit.*, p. 463.
[2] *loc. cit.*, p. 462.

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[*Reprinted from the Journal of Physiology.*
Vol. XXXII. Nos. 5 *&* 6, *July* 13, 1905.]

THE CHANGES IN THE VISCOSITY OF THE BLOOD DURING NARCOSIS[1]. By R. BURTON-OPITZ.

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THE question, whether the narcosis produces noticeable changes in the viscosity of the "living" blood, has been answered by me in part in an earlier paper[2]. I determined at that time the viscosity of the blood in a rabbit before and after the administration of morphine and a mixture of chloroform and ether. This experiment showed unmistakably that the viscosity of the blood is not altered by the narcosis. If any changes did result in the course of this experiment, they were too insignificant to be recognized by the method employed.

The determinations to be reported in this paper, concern the changes in the viscosity of the blood which occur during light and deep narcosis produced by morphine and ether or by morphine and chloroform. The experiments were performed upon dogs in accordance with the method described by Hürthle[3].

The coefficients expressing the viscosity are obtained by calculation. For this purpose it is essential to know the following factors: the quantity of blood collected during a definite period, its specific gravity, the blood-pressure, and the length and internal diameter of the capillary tube. The values thus obtained are subsequently compared with the coefficient of the viscosity of distilled water at 37° C. which, according to Poiseuille, is 4700. Hence, the viscosity of the blood is expressed in this investigation in terms of multiples of the aforesaid coefficient (denoted by K) for distilled water at 37° C.

Two capillary tubes, A and B, were employed in these experiments. The former measured 245·5 mm. in length, the latter 213·0 mm. The

[1] This study was conducted under a grant from the Rockefeller Institute for Medical Research.

[2] Burton-Opitz, R., *Archiv für die ges. Physiol.* LXXXII. p. 448. 1900.

[3] Hürthle, K., *Archiv für die ges. Physiol.* LXXXII. p. 415. 1900. A brief description of the method is also given in this *Journal*, XXXII. p. 8.

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internal diameter of *A* was 0·6636 mm.; that of *B* 0·66362 mm. The accuracy of these tubes was tested by a number of preliminary experiments with distilled water. In order that the unavoidable variations between the different determinations might be reduced to a minimum, only one capillary was used during a given experiment.

The method followed in each experiment was as follows:—Excepting two, all the animals to be used received small doses of morphine, one half-hour before the first determination began. They were then narcotized either with ether or with chloroform. It must be pointed out, however, that the degree of narcosis was not kept the same, but was changed after each determination. Thus, if the first determination had been made during deep narcosis, the second determination was made during light narcosis, and so on. The different determinations were separated by intervals of from ten to fifteen minutes, during which the capillary tube was cleaned and readjusted. The corneal reflex served as an indicator of the depth of the narcosis.

The results of the experiments are given in detail in Tables I and II. The first table represents five experiments with ether, and the second three experiments with chloroform. The different determinations of each experiment are numbered and are marked in addition with the letters *D* and *L*, indicating thereby whether the determination was made during deep (*D*) or during light (*L*) narcosis. Among other details, the tables also contain the weight of the animals, the amount of morphine administered, the rectal temperature at the beginning of the experiment and the specific gravity of the blood. The latter was obtained by means of small pycnometers during deep as well as during light narcosis. As many as four determinations of the specific gravity were made in the course of each experiment.

When the coefficients given in these tables are compared with one another, it is seen that their value changes in a very precise and regular manner. They become larger when the narcosis is lessened, and smaller when the narcosis is deepened. In other words, the viscosity of the blood does not remain constant during narcosis. It is increased by deep and lessened during light narcosis. Naturally, perfectly regular variations could not be obtained, no method existing by means of which the degree of narcosis could be rendered absolutely equal during two alternate determinations.

Considered collectively, these differences in the viscosity during light and deep narcosis cannot be considered of great importance, and the changes which result from the administration of moderate doses of

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TABLE I. *The Viscosity of the Blood during Ether Narcosis.*

Exp.	Number of determination	Cap.	Weight of dog and of morphine	Degree of Narcosis	Temperature 0° C.	Specific Gravity of blood	Quantity escaped from cap., mg.	Time sec.	Pressure mm. Hg.	Viscosity coefficient K	Difference between successive determinations	Mean value of K
I.	1	A	12 Kg.	D	36·9	—	1194·6	10·63	132·40	1017·18	45·82	1021·64
	2	,,	0·06 g.	L	,,	1·05558	1749·2	13·85	142·50	1063·00	70·10	
	3	,,		D	,,	1·05652	1791·3	18·40	117·50	992·90	53·35	
	4	,,		L	,,	1·05618	2035·8	16·59	140·60	1046·25	57·34	
	5	,,		D	,,	1·05698	1523·8	15·37	120·20	988·91		
II.	1	A	10 Kg.	L	37·0	1·05635	1834·1	12·54	192·30	911·62	54·66	858·55
	2	,,	no morph.	D	,,	1·05782	3026·4	20·74	203·80	856·96	19·92	
	3	,,		L	,,	—	2063·9	14·40	195·90	876·88	88·11	
	4	,,		D	,,	—	2319·4	18·76	187·60	788·77		
III.	1	A	10 Kg.	D	37·0	1·05689	1647·8	16·63	126·00	942·05	55·01	969·55
	2	,,	0·05 g.	L	,,	1·05580	2259·9	17·49	155·40	997·06		
IV.	1	B	12 Kg.	D	36·6	1·06235	2095·2	15·89	190·40	716·00	33·71	715·77
	2	,,	0·05 g.	L	,,	1·06205	2560·1	17·64	200·20	749·71	39·82	
	3	,,		D	,,	1·06230	1988·2	15·67	184·80	709·89	37·65	
	4	,,		L	,,	1·06215	2670·1	18·74	197·10	747·54	64·69	
	5	,,		D	,,	—	1971·6	15·99	186·70	682·85	5·81	
	6	,,		L	,,	—	2044·9	15·82	194·10	688·66		
V.	1	B	7 Kg.	D	36·5	1·05236	1721·1	19·05	99·80	944·83	37·69	958·45
	2	,,	0·05 g.	L	,,	1·05162	1711·2	18·19	100·00	982·52	47·82	
	3	,,		D	,,	1·05218	1471·6	16·24	101·20	934·70	17·07	
	4	,,		L	,,	1·05200	2396·4	17·87	147·10	951·77		

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TABLE II. *The Viscosity of the Blood during Chloroform Narcosis.*

Exp.	Number of determination	Cap.	Weight of dog and of morphine	Degree of Narcosis	Temperature 0° C.	Specific Gravity of blood	Quantity escaped from cap., mg.	Time sec.	Pressure mm. Hg.	Viscosity coefficient K	Difference between successive determinations	Mean value of K
I.	1	B	14 Kg.	L	37·0	1·04650	2046·5	14·05	119·20	1282·52	94·70	1242·14
	2	,,	0·05 g.	D	,,	1·04520	1380·5	14·59	83·70	1187·82	103·30	
	3	,,		L	,,	—	2102·8	13·47	126·90	1291·12	64·54	
	4	,,		D	,,	—	1585·0	16·93	80·20	1226·58	14·34	
	5	,,		L	,,	—	2672·1	17·52	129·00	1240·92	17·02	
	6	,,		D	,,	—	1457·6	18·43	67·90	1223·90		
II.	1	B	8 Kg.	D	36·8	1·06370	719·1	14·66	77·08	657·10	36·05	702·46
	2	,,	no morph.	L	,,	1·06500	950·1	7·58	186·50	693·15	3·11	
	3	,,		D	,,	1·06475	726·2	11·50	94·40	690·04	80·18	
	4	,,		L	,,	—	1512·6	13·00	155·80	770·22	68·41	
	5	,,		D	,,	—	877·9	11·87	108·70	701·81		
III.	1	B	14 Kg.	D	36·9	1·05942	1422·8	14·93	121·20	815·10	15·37	802·32
	2	,,	0·05 g.	L	,,	1·05952	1017·9	20·56	61·80	830·47	60·87	
	3	,,		D	,,	—	2085·8	25·20	111·50	769·60	47·82	
	4	,,		L	,,	—	1634·0	18·27	113·70	815·42	34·38	
	5	,,		D	,,	—	1375·5	25·50	71·60	781·04		

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morphine and ether or morphine and chloroform must at most be extremely slight. In only a few instances did the difference amount to more than sixty points. For example, in experiment 3, Table I, the coefficient amounted to 997 during light and 942 during deep narcosis. This implies that, in this case, the viscosity of the blood during light narcosis was 4·7 times as great, and during deep narcosis 5·0 times as great as that of distilled water at 37°.

The specific gravity of the blood also showed regular variations. The specific gravity was increased by deep and lessened by light ether narcosis; the changes in the specific gravity pursue in this case a parallel course to those in the viscosity. During narcosis from chloroform the inverse relationship was found to exist, a slight decrease in the specific gravity being noticeable during deep chloroform narcosis; hence the specific gravity cannot be regarded as a perfectly sure index of the viscosity.

The mean value of K obtained from the eight experiments reported is 908. The mean viscosity of dog's blood is, therefore, 5·5 times as great as that of distilled water at 37° C.

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The

Journal of Infectious Diseases

FOUNDED BY THE MEMORIAL INSTITUTE FOR INFECTIOUS DISEASES

VOL. II *January 12, 1905* No. 1

SOME PHYSICAL PROPERTIES OF ENZYMES.*

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INTRODUCTION.

THE fact that enzyme solutions decrease in activity as the result of filtration has been frequently observed. Musculus[1] in 1874 called attention to the retention of the enzyme urase by filter paper, a fact which Lea[2] explained by the assumption that urase is not a soluble ferment. Miquel,[3] however, demonstrated the solubility of this enzyme.

*Received for publication August 4, 1904.

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Wortmann[4] in 1890 attempted to demonstrate the presence of a ferment in green leaves. He tested the filtered aqueous extracts of crushed leaves for diastatic activity, and the leaves whose filtrates did not invert starch he declared free from diastase. This work elicited from Brown and Morris[5] (1893) the following:

The absence of diastase from a clear filtrate obtained after merely digesting the crushed leaves with water for three or four hours is no sufficient proof that the leaf does not contain a starch-transforming enzyme; for it is well known to those who have spent much time in separating such enzymes from vegetable or animal tissues that the protoplasm often parts with its enzyme with difficulty, and that it is often almost impossible to obtain from such a preparation a clear filtrate which has any hydrolyzing action, although an energetic hydrolysis may be produced by contact with the tissue itself, or by the employment of a turbid filtrate containing the finely divided tissue in suspension.

They cite in evidence the following experiment:

The leaf of Helianthus tuberosus was extracted by the prolonged action of water upon the crushed and pounded fresh leaf. The influence of microorganisms was guarded against by the use of chloroform. The action of the filtered extract was then compared with a like maceration of the leaves themselves. The diastatic activity of 10 g. of the dried leaf of Helianthus, acting by contact, was found to be 3.78, while that of the filtered extract of 10 g. of the same leaf was 0.53.

They refer, further, to the observation of Brown and Herron[6] (1880) that the clear, filtered, aqueous infusion of tissue of the small intestine of the pig had but slight hydrolytic action on starch, whereas contact with the tissue itself produced pronounced hydrolysis.

Vines[7] (1891) found that the turbid extract of green leaves when merely strained was much more active than the clear extract obtained by filtration.

Kastle and Loevenhart,[8] in their work on lipase, macerated the pancreas with sand, then mixed the mass with water, and simply strained through linen or cotton cloth. They report:

The effect of filtration was invariably to diminish the activity of the solution. This was proved experimentally for aqueous and glycerin extracts of both pancreas and liver. The same was also found to hold for the pancreatin of Parke, Davis & Co. A two per cent. solution of this preparation is yellowish in color and is turbid. In this shape it possesses weak but measurable lipolytic power. On filtering a great many times, a clear yellowish solution is obtained, which possesses only a trace of its original lipolytic activity.

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To show further the effect of filtration on a liver extract, they cite the following:

Before filtering, 1 c.c. of a 10 per cent. liver extract was found to hydrolyze 6.28 per cent. of ethyl butyrate in fifteen minutes at 40°. After filtering repeatedly through the same filter paper, it hydrolyzed only 2.76 per cent., showing a falling off in activity of over one-half as the result of filtration. The removal of lipase along with suspended matter in the solution was observed in still another connection. When an active pancreatic extract acts on ethyl butyrate at 40°, the butyric acid produced soon coagulates the proteids in the extract. On filtering it was found that the filtrate possessed little or no lipolytic power, whereas the proteid coagulum was found to be exceedingly active.

PAPER FILTRATION OF PTYALIN.

In order to determine whether or not ptyalin could be removed from saliva by filtration through paper, the following experiment was carried out:

Two hundred cubic centimeters of saliva were collected by mastication of paraffin, and subjected to filtration through eight double filters of Schleicher and Schüll's hardened filter paper, No. 575. These filters were arranged as follows: In each of two funnels were placed two folded filter papers. The funnels were arranged one above the other on a ring stand, so that the stem of the upper funnel touched the side of the filter paper in the lower one. The stem of the lower one passed into an Erlenmeyer flask. Thus the saliva poured into the upper funnel passed through four thicknesses of paper before reaching the flask. This filtrate, designated as "2 × 2," then passed through a second set of filters similarly arranged and gave filtrate "4 × 2," the passage of which through another set of double filters produced filtrate "6 × 2," which in turn filtered as before, through a fourth set of new double papers, gave as final filtrate, "8 × 2." In all of our paper-filtration experiments, the enzyme solution was filtered through eight double filters of hardened paper.

Previous to the filtration through the eight double filters, the saliva was filtered through one thickness of ordinary filter paper (S. & S., No. 595) to remove the mucin and solid particles which otherwise retarded the subsequent filtration.

In order to ascertain the decrease in diastatic activity, a definite quantity of each filtrate was added to a definite volume of a starch solution, and the resulting mixture was kept at a fixed temperature in a water bath. From time to time a portion of the liquid was removed and tested with iodine until the achromic point was reached.

A preliminary trial indicated the necessity of using but small portions of the successive filtrates in order to determine the pro-

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gressive decrease in activity of the filtered solution, since in concentrated solutions the action of ptyalin is evidently not directly proportional to the amount of the enzyme present. The following experiment will make this clear:

Two hundred cubic centimeters of saliva, previously passed through one thickness of ordinary filter paper, were subjected, as above described, to eight successive double filtrations; each filtrate and a portion of unfiltered saliva which was set aside as control, were tested by adding 0.5 c.c. of each to 30 c.c. of a 1 per cent. solution of starch, and the mixture was allowed to digest at 34–37°. A similar set was made, differing only in that the control and each filtrate were tested by adding 0.1 c.c. of each to 30 c.c. of a 1 per cent. starch solution. The filtration of the first set consumed about two hours, while that of the second required three and one-quarter hours. The following table gives the results, indicating the number of minutes required in each test to reach the achromic point:

TABLE I.

	Digested with 0.5 c.c.	Digested with 0.1 c.c.
Control	10 min.	30 min.
"2 × 2" filtrate	10 min.	42 min.
"4 × 2" filtrate	10 min.	50 min.
"6 × 2" filtrate	8 min.	62 min.
"8 × 2" filtrate	6 min.	Pink in 82 min.

The above, then, indicates that, although ptyalin is removed from its solution by filtration, this fact is not made manifest unless the quantity of saliva employed in testing is small enough to prevent the masking of the loss by the activity of the remaining enzyme. For this reason in the following experiments diluted saliva was employed.

EXPERIMENT I.

Twenty cubic centimeters of saliva were collected by mastication of paraffin and filtered through one thickness of ordinary filter paper. This filtrate was diluted to 200 c.c. with distilled water. Filtration was then carried out as above described. A portion of the original diluted saliva was set aside (10 per cent. solution) in a test-tube at the same temperature and light conditions as obtained for the filtering saliva. One cubic centimeter of this control solution was added to 30 c.c. of the starch solution at the same time that 1 c.c. of each successive filtrate was similarly treated. A filtered 1 per cent. starch solution containing glycerin was employed for these tests. The filtering liquid and the control were protected from light by black cloth. The progress of digestion in each case was noted by removing from each tube from time to time 2 c.c. of the solution and testing this with a dilute

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iodine solution. The pipette used was rinsed thoroughly each time with boiling distilled water.

The time required to reach the achromic point is indicated in the following table:

TABLE II.

1 C.C. 10 PER CENT. SALIVA + 30 C.C. OF STARCH SOLUTION, DIGESTED AT 34°.

Control	Achromic Point	Filtrate from	Achromic Point
"2 × 2"	20 min.	"2 × 2"	20 min.
"4 × 2"	20 min.	"4 × 2"	20 min.
"6 × 2"	20 min.	"6 × 2"	25 min.
"8 × 2"	20 min.	"8 × 2"	Deep purple in 1 hr. 45 min.

The time consumed in filtration was two hours and ten minutes.

TABLE III.

0.5 C.C. SALIVA + 30 C.C. OF STARCH SOLUTION, DIGESTED AT 34°.

(This experiment is like the preceding save that 0.5 c.c. of control and of each filtrate was added to 30 c.c. of the starch solution.)

Control	Achromic Point	Filtrate from	Achromic Point
"2 × 2"	40 min.	"2 × 2"	48 min.
"4 × 2"	40 min.	"4 × 2"	54 min.
"6 × 2"	36 min.	"6 × 2"	70 min.
"8 × 2"	36 min.	"8 × 2"	Blue in 1 hr. 30 min. Purple in 18 hrs.

The duration of the filtration process in this case was two hours and thirty minutes.

Further experiments gave the same results as the preceding, showing without a doubt that saliva is relieved of its ptyalin by filtration through hardened filter paper.

To what, then, is this result due? Three answers present themselves: the molecules of ptyalin, as in the case of a precipitate, may be mechanically retained by the filter; or the aeration or the surface action, if any, may have a destructive effect; or, finally, the ptyalin may have entered into firm combination with the material of the filter paper, *i. e.*, it may have been *fixed.*

EXPERIMENT III. (BERKEFELD FILTRATION.)

To determine whether the paper acted by mechanical retention, the following experiment was carried out:

A.—20 c.c. of saliva, filtered through a single ordinary filter paper, were diluted to 200 c.c. with distilled water. The entire quantity, less 5 c.c. which were set aside as control, was filtered through a No. 5 Berkefeld filter (1 × 8

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inches) at room temperature. The diastatic action of the filtrate was tested and compared with that of the control solution.

B.—Same as A, save that filter was warmed to 50° by repeated washings with distilled water warmed to 50° and 195 c.c. of 10 per cent. saliva warmed to 50° filtered through bougie. Control of unfiltered saliva warmed to 50°. Tested filtrates and controls as before. The time required to produce achromicity is indicated in the table below:

TABLE IV.

0.5 C.C. OF SALIVA + 30 C.C. STARCH SOLUTION, AT 34°.

	Temperature	Filtration Pressure	Rate of Filtration	Achr. Pt. of Filtrate	Achr. Pt. of Control
A.....	Room	740 mm.	7.5 min. per 100 c.c. *ca.*	40 min.	40 min.
B.....	50°	720 mm.	7 min. per 100 c.c. *ca.*	40 min.	40 min.

This disposes of the idea of the mechanical retention of the molecules. It is quite inconceivable that the molecules should be retained by the comparatively large pores of the filter paper, and be allowed unimpeded passage through the pores of the Berkefeld filter which denies passage to minute bacteria. Some factor other than mechanical retention has been at work.

EXPERIMENT IV.

To determine the effect of aeration the following experiment was performed:

Over the narrowed end of a piece of amber glass tubing 46 cm. long and 1½ cm. wide was slipped a piece of rubber tubing 5 cm. long. This tube was provided with a capillary glass tip and with a Hofmann clamp. The amber glass tube was then filled to a depth of about 35 cm. with clean glass beads, which were prevented from escaping from the tube by a thin plug of glass wool. The tube was then clamped in an upright position to a ring stand. The Hofmann clamp was closed, and about 25 c.c. of 10 per cent. saliva (filtered through ordinary filter paper before dilution) were poured into the tube. A rubber stopper carrying an L-shaped glass tube closed the upper end of the tube, and this L was connected by rubber tubing with an aspirating pump. Suction was started and the Hofmann clamp was opened sufficiently to allow a vigorous, but not too violent, aeration of the contents of the tube. This aeration was allowed to continue for twenty-four hours, the amylolytic action of the liquid being tested before aeration was begun, also at the end of three, six, nine, and twenty-four hours respectively. A control of unaerated 10 per cent. saliva set aside at room temperature under a black cloth was likewise

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tested at the same intervals. Saliva, control and aerated, was tested by adding 0.5 c.c. to 30 c.c. of 1 per cent. starch solution, and digestion was allowed to proceed at 35°. The time required to produce achromicity is indicated in the following table:

TABLE V.

Control	Achromic Point	Saliva Aerated for	Achromic Point
Original...	32 min.	.....	.....
3 hrs.....	20 min.	3 hrs.....	24 min.
6 hrs.....	22 min.	6 hrs.....	24 min.
9 hrs.....	38 min.	9 hrs.....	38 min.
24 hrs.....	24 min.	24 hrs.....	24 min.

The slowness of digestion in the nine-hour set was probably due to the fact that the digestion temperature was 32°, whereas that of the other sets was 35°.

It is evident from the above that aeration, even for twenty-four hours, is without appreciable injurious effect on a 10 per cent. saliva.

Szumowski[9] has demonstrated the fixation of enzymes (ptyalin, rennin, pepsin, trypsin) by fibrin, and Effront[10] states that enzymes are fixed by different substances, such as silk and fibrin. Chevreul[11] (1853) tested the fixing or mordant action of wool, cotton, and silk on such substances as sodium chloride, sulphuric acid, mercuric chloride, hydrochloric acid, lime water, baryta water, alum, barium nitrate, lead nitrate, and ferrocyanide of potassium. To quote from one experiment:

> Thus cotton absorbs $HgCl_2$ and water in the proportion of the solution, and moreover, it retains a portion of it with so much force that, after having been washed until the wash-water no longer precipitates $AgNO_3$, it is colored by a solution of H_2S and it acts otherwise than pure cotton on the coloring principles of cochineal, logwood, and madder.

"The action of solids in altering solutions," says Chevreul, "shows how filters can act in a chemical manner on the liquids which traverse them."

Musculus (*loc. cit.*) shows that if urine in active alkaline fermentation be filtered through paper, this paper, after being washed until the wash-water is free from any alkaline reaction and dried at 35 or 40°, retains, even for months, a something capable of exciting alkaline fermentation of urea. This something he considered to be "torulæ," retained in the pores of the

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paper. It is possible, in view of the demonstration by Miquel (*loc. cit.*) of the solubility of urase, that the filtered paper had fixed the urase.

O'Shea[23] found that lead was retained by various filter papers (English, French, and German make) on filtering lead salts. An experiment shows that a S. & S. filter, 589, absorbed from 50 c.c. of lead acetate 0.12 mg. of lead, 0.02 mg. of which was removed by a first and none by a second washing. On refiltering 0.24 mg. was absorbed, none of which was removed by subsequent washing.

That hardened filter paper fixes ptyalin is proved by the following experiments:

EXPERIMENT V.

In the aeration tube above described were placed about 1,500 sq. cm. of hardened filter paper, cut into bits about ½ cm. sq. and then crumpled. The papers in the tubes were first washed with distilled water and then with 10 per cent. saliva (filtered through ordinary filter paper before dilution), after which 35-40 c.c. of the 10 per cent. solution were added and aerated over the paper bits, the same as before over beads, and the diastatic activity was tested at intervals of three, six, nine, and twenty-four hours. A portion of unaerated saliva was set aside at room temperature under a black cloth for control and was tested at the same intervals.

The time required to produce achromicity is shown in the following table:

TABLE VI.

0.5 C.C. 10 PER CENT. SALIVA + 30 C.C. 1 PER CENT. STARCH SOLUTION AT 35°.

Control	Achromic Point	Saliva Aerated for	Achromic Point
Original.....	36 min.	.....	.....
3 hrs.....	36 min.	3 hrs.....	64 min.
6 hrs.....	32 min.	6 hrs.....	60 min.
9 hrs.....	32 min.	9 hrs.....	56 min.
24 hrs.....	32 min.	24 hrs.....	60 min.

Although aeration over beads produced no change in activity of saliva, aeration over paper did. The saliva was now drained off, and the papers were washed by rinsing and aerating with distilled water repeatedly, until the wash-water no longer exhibited a trace of ptyalin.

A number of shreds of paper were then removed from the tube, rinsed in a beaker of water, and added to 30 c.c. of a 0.1 per cent. starch solution. To another tube containing 30 c.c. of the starch solution was added an equal quantity of fresh crumpled bits of hardened filter paper, washed in distilled water. This latter served as control. Both were placed in a thermostat at 35°. Portions of contents of each tube responded to iodine with blue coloration at the end of an hour. Absence from laboratory prevented further test until at the end of forty hours, at which time the *starch in the tube*

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which contained paper aerated with saliva gave achromic reaction with iodine, while starch in control tube showed no inversion whatever.

At this time the water was drawn off from the washed papers which remained in the tube, and glycerin was added. Aeration was then proceeded with for thirty minutes, at the end of which time the glycerin was withdrawn and 10 c.c. added to 10 c.c. of the 0.1 per cent. starch solution. This was placed in water bath at 35°, and portions were tested with iodine at intervals indicated below, with the following results:

At the end of fifteen minutes the color produced by the iodine was blue. When next tested at the end of twenty-five minutes, no color reaction was obtained. This shows that, as will be borne out in subsequent experiments, the glycerin had extracted the ptyalin which had been retained by the filter paper.

EXPERIMENT VI.

The saliva was aerated over paper as in the preceding experiment. The aerated saliva and control were tested at the end of three hours and fifteen and one-half hours.

TABLE VII.

0.5 C.C. OF 10 PER CENT. SALIVA + 30 C.C. OF 1 PER CENT. STARCH SOLUTION AT 35.5°.

Control	Achromic Point	Saliva Aerated for	Achromic Point
3 hrs.....	20 min.	3 hrs.....	20 min.
15½ hrs.....	20 min.	15½ hrs.....	20 min.

In the preliminary filtration experiments it was seen that, although the liquid experimented with loses some of its ptyalin, the activity of the remaining ptyalin hides this fact. That this is also the case with saliva aerated in Experiment VI is shown by the following:

The paper bits in the tube were washed with distilled water until all traces of free ptyalin were removed, and the paper was then taken from the tube. A number of the bits were added to 30 c.c. of a 0.1 per cent. starch solution and placed in the thermostat at 36°. A corresponding number of fresh bits were likewise added to 30 c.c. of starch solution for control. The experiment was further controlled by placing in the thermostat a third tube containing 15 c.c. of the last wash-water, added to 15 c.c. of the starch solution. Portions of the liquid in each tube were tested from time to time with iodine, with the following results:

Control wash-water + starch solution — blue in 2½ hrs.
Control paper + starch solution — blue in 2½ hrs.
Paper aerated with saliva + starch solution — achromic in 2½ hrs.

At the end of nineteen hours both controls gave purple reaction with iodine.

EXPERIMENT VII.

The bits of paper from Experiment VI not employed in making the above test were kept in refrigerator for twenty-one hours. They were then returned to the washed tube, which was labeled *A*. In another similar tube, labeled

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B, was placed an approximately equal amount (about 1,000 sq. cm.) of paper in crumpled bits. To each tube was added 70 c.c. of 10 per cent. saliva, filtered before dilution through ordinary filter paper. The contents of the tubes were then aerated side by side at equal rates of aeration for twenty-four hours, and both were controlled by an unaerated 10 per cent. saliva kept at room temperature under a black cloth. The control was tested at the same intervals as the aerating saliva. The saliva was extremely active, and for that reason in the following tests only 0.1 c.c. of saliva was employed.

TABLE VIII.

0.1 C.C. SALIVA + 30 C.C. STARCH SOLUTION AT 36°.

Tube *A* contained old saliva papers.
The *B* contained fresh saliva papers.

	Control Saliva	Saliva from *A*	Saliva from *B*
3 hrs.....	Achromic in 60 min.	Achromic in 64 min.	Achromic in 66 min.
6 hrs.....	Achromic in 60 min.	Achromic in 74 min.	Achromic in 74 min.
24 hrs.....	Pink in 80 min.	Achromic in 66 min.	Achromic in 66 min.

The starch used in testing the twenty-four-hour control was twenty-four hours older than that used in testing the twenty-four-hour sets of *A* and *B*, which may account for the irregularity apparent in twenty-four-hour set.

This experiment was performed for the purpose of a comparison of papers presumably ptyalin-saturated (*A*), with fresh papers (*B*) as regards their action on 10 per cent. saliva. Both appear to have similar effect, a slightly greater avidity for ptyalin being suggested by the fresh papers at the end of three hours, after which they too appear to be saturated. A better comparison is afforded in Table XIII.

The paper bits in both tubes were now thoroughly washed until no trace of free ptyalin remained. The action of the papers was then tested as before.

TABLE IX.

Control	Iodine Reaction	Papers from	Iodine Reaction
Fresh paper control	Blue in 1 hr.		
Wash-water control (*A*).....	Blue in 1 hr. Red in 2 hrs. Achromic in 19 hrs.	*A* (old saliva papers)	Achromic in 1 hr.
Wash-water control (*B*).....	Blue in 2½ hrs.	*B* (fresh saliva papers)	Achromic in 1 hr.

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In the meantime the papers in *B* were extracted by aeration with glycerin for thirty minutes. The test of the aerated glycerin for diastatic activity as compared with fresh glycerin resulted as follows:

15 c.c. of paper aerated glycerin + 15 c.c. 0.1 per cent. starch solution produced achromicity in 18 hours.

15 c.c. of fresh glycerin + 15 c.c. of 0.1 per cent. starch solution produced no inversion whatever in 18 hours.

EXPERIMENT VIII.

Added fresh glycerin to papers in *B* and fresh distilled water to *A*, and aerated for twenty-four hours. Tested wash-water of *A* and glycerin of *B* as before, controlling with fresh water and fresh glycerin.

TABLE X.

15 C.C. OF WATER AND OF GLYCERIN + 15 C.C. 0.1 PER CENT. STARCH SOLUTION AT 36°.

Control	Color Produced by Iodine	Wash Liquid	Color Produced by Iodine
Water + starch	Deep purple in 4½ hrs.	Water (*A*)	Deep purple in 4½ hrs.
Glycerin + starch ..	Blue in 1 hr.	Glycerin (*B*)	Achromic in 1 hr.

Portions were tested every fifteen minutes in the above tests. Thus with twenty-four hours' washing, water removed none of the retained ptyalin whereas glycerin extracted it.

The tubes were then drained and rinsed with distilled water, after which fresh water was added to *A* and fresh glycerin to *B*. They were aerated then for forty-eight hours, and tested as before.

TABLE XI.

Control	Color Produced by Iodine	Wash-Liquid	Color Produced by Iodine
Water + starch	Blue in 4½ hours	Water (*A*)	Blue in 4½ hours
Glycerin + starch ..	Blue in 4½ hours	Glycerin (*B*)	Achromic in 4½ h.

Even this attempt at extraction (seventy-two hours' aeration) with water failed to remove the ptyalin from its close retention by the filter paper, although the paper yielded its ptyalin readily to the glycerin. That the paper still retained its ptyalin is further shown by the following:

The glycerin was thoroughly removed from *B* by repeated washings with distilled water. *A* was drained, and a third control tube was set up, containing fresh papers, approximately equal in quantity to those in *A* and *B*. Water was placed in tubes *A*, *B*, and *C*, and all were aerated for one and three-quarter

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hours. A portion of the papers was then removed from *A* and *B*, and the amylolytic action tested; tests were controlled by paper bits from *C*. Controlled further with wash-water from *A* and *B* by adding 15 c.c. of each to 15 c.c. of 0.3 per cent. starch solution and digesting at 36°.

TABLE XII.

Control	Color Produced by Iodine	Saliva Paper	Color Produced by Iodine
Paper control.....	Blue in 3½ hrs. Purple in 18 hrs.		
			Blue in 3½ hrs.
Wash-water of *A*..	Blue in 3½ hrs. Purple in 18 hrs.		Purple in 6 hrs.
		A (water-washed)	Achr. in 18 hrs.
Wash-water of *B*..	Blue in 3½ hrs. Purple in 18 hrs.		
		B (glyc. extracted)	Purple in 3½ hrs. Achr. in 18 hrs.

The liquids were examined only at the times indicated in the table.

After enough liquid was removed from each tube for the preceding test, the tubes and contents were rinsed with 2 per cent. saliva (the latter being filtered, before dilution, through a single thickness of ordinary filter paper). To each tube there were then added 25 c c. of the 2 per cent. saliva, and the contents were aerated for twenty-four hours, with interruptions at the end of three and six hours as before. The tubes contained approximately equal quantities of paper and were aerated side by side at equal rates.

Tube *A* contained old saliva papers, water-washed.

Tube *B* contained old saliva papers, glycerin-extracted.

Tube *C* contained fresh papers, water-washed.

At the end of three, six, and twenty-four hours, 1 c.c. aerated 2 per cent. saliva from each tube was added to 30 c.c. starch solution and digested at 34° to 37°. The results are tabulated below:

TABLE XIII.

Aeration	Color Produced by Iodine			
	Control	Tube *A*	Tube *B*	Tube *C*
3 hours.....	Achr. in 50 min.	Achr. in 7 hrs.	Achr. in 6 hrs.	Blue in 7 hrs.
6 hours.....	Achr. in 50 min.	Pink in 8 hrs.	Achr. in 6½ hrs.	Blue in 1¼ hrs.
24 hours....	Achr. in 50 min.	Blue in 3 hrs.	Purple in 3 hrs.	Blue in 3 hrs.

It is seen from the above that with sufficient dilution of saliva all of the ptyalin can be retained by the filter paper in the aeration tubes, just as it can be removed from like solutions by filtration. The paper bits in *A* and *B* already contained fixed ptyalin,

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and on that account could not remove all the ptyalin from the 2 per cent. saliva, although a glance at the table shows that a very considerable quantity was removed. The paper in *C* so effectually removed the ptyalin that at the end of three hours' aeration 1 c.c. of the liquid could produce no effect on 30 c.c. of 1 per cent. starch solution in seven hours, whereas a corresponding quantity of the control saliva completely inverted 30 c.c. of the starch solution in fifty minutes.

That the ptyalin was not destroyed is made evident by the following:

The paper bits in Tubes *A*, *B*, and *C* were thoroughly washed as before and approximately equal quantities of each were immersed in test-tubes containing 30 c.c. of 1 per cent. starch solution. Control tests were made with fresh paper as before. Wash-water controls as before. The tests with the wash-waters were checked with distilled water. The digestion was carried on at 35°.

TABLE XIV.

Water Control	Color Produced by Iodine	Paper Bits	Color Produced by Iodine
Distilled water.....	Blue in 18 hrs.	Control.....	Purple in 18 hrs.
Wash-water *A*.....	Blue in 18 hrs.	Tube *A*.....	Pink in 3½ hrs. Achromic in 18 hrs.
Wash-water *B*.....	Blue in 3½ hrs. Pink in 18 hrs.	Tube *B*.....	Achromic in 3½ hrs. Blue in 1 hr.
Wash-water *C*.....	Blue in 18 hrs.	Tube *C*.....	Pink in 3½ hrs. Achromic in 18 hrs.

The liquids were examined only at times indicated in the table.

EXPERIMENT IX.

Two sheets of S. & S. hardened filter paper, No. 575, diameter 24 cm., were cut up into small bits (about 1 cm. square), crumpled, and placed in an aeration tube, which we will designate as *A*. A similar quantity of papers was placed in a second tube *B*. In a third tube *C* were placed glass beads, the height of the column of which equaled that of the paper in *A* and in *B*. The saliva was filtered through a single thickness of ordinary filter paper and 4 c.c. of it was diluted to 200 c.c. with distilled water. After rinsing the tubes with this 2 per cent. saliva, 60 c.c. of the same were placed in *A* and *B* respectively, while *C* received 40 c.c.

Tubes *A* and C were connected with the pump and aerated at the same rate. Tube *B* remained unaerated for two hours, and was then aerated for five minutes, after which it remained unaerated for two hours, and then was again aerated for five minutes. It now remained unaerated for the next eighteen hours, after which it was aerated for fifteen minutes. The saliva in

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A, *B*, and *C* was tested at three-hour intervals, and the tests were controlled with unaerated 2 per cent. saliva, which was kept at room temperature under a black cloth.

TABLE XV.

1 C.C. OF 2 PER CENT. SALIVA + 30 C.C. 1 PER CENT. STARCH SOLUTION AT 38°.

(For the six-hour test a new starch solution was prepared.)

SALIVA	COLOR PRODUCED BY IODINE		
	3 Hours	6 Hours	24 Hours
Control.....	Achr. in 26 min.	Achromic in 50 min.	Achromic in 38 min.
A.....	Achr. in 62 min.	Deep purple in 1 hr.; achr. in 16½ hrs.	Purple in 3 hrs.; achr. in 4 hrs.
B.....	Achr. in 62 min.	Blue in 1 hr.; achr. in 16½ hrs.	Blue in 4 hrs.; deep purple in 24 hrs.
C.....	Achr. in 26 min.	Achr. in 50 min.	Achr. in 32 min.

From the above table it will be seen that aeration over beads has no effect, and that fixation is more rapid when the liquid is not continuously agitated.

The papers in *A* and *B* were then washed by aerating for twenty-four hours with distilled water, which was frequently changed. The inverting action of the washed papers was tested as before.

TABLE XVI.

Paper Bits	Color Produced by Iodine
Control.....	Blue in 5¼ hrs.
Saliva paper *A*...	Blue in 2 hrs.; light pink in 5¼ hrs.
Saliva paper *B*...	Blue in 2 hrs.; achromic in 5¼ hrs.

DIALYSIS OF PTYALIN THROUGH COLLODION SAC.

Gorsline,[12] working in this laboratory, showed that such substances as starch and albumin can pass through the collodion membrane, and suggested the possibility of dialyzing enzymes. According to Rodet and Guéchoff,[13] the collodion wall does not act as a perfect filter. They say:

To be acquainted with the value of collodion sacs in bacteriological experiments it is quite necessary to know whether the membrane of collodion constitutes a filtering septum, retaining only formed particles, and allowing to pass indiscriminately all soluble matters, or whether, endowed with properties intermediate between those of such a filtering wall and those of a semipermeable membrane, it allows soluble substances to pass more or less, and in a varying degree, according to their nature and the size of their molecules.

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We have wished first to see by an experiment *in vitro* if collodion is perfectly permeable to bodies of different molecular weights, such as sugar, peptone, albumin. . . . . Three similar sacs were filled with distilled water, and were plunged into test-tubes containing several cubic centimeters, one of a solution of glucose, another of a two per cent. aqueous peptone solution, and the third of blood serum. After twenty-four hours' sojourn at laboratory temperature special reactions were noticed in the contents of the sacs. The contents of the first reduced Fehling's solution abundantly; the sugar had traversed the wall of the collodion. The third sac gave the albumin reactions; but the content of albumin was very feeble, estimated practically 500 times less than that of the serum in which the sac was immersed; the albumin had traversed the wall but feebly. As to the contents of the second sac, the peptone reaction was not found there; it is, however, quite reasonable to suppose that it had passed, but in a quantity insufficient to be detected by the reagents. This experiment shows that collodion does not comport itself as a perfect filter.

It is quite reasonable to suppose that the sacs used by Messrs. Rodet and Guéchoff in this experiment were not of the same degree of thickness—that the sac used in the peptone dialysis was thicker than that used in the dialysis of albumin. If albumin readily traverses the collodion wall, it is quite evident that the relatively simpler peptone should do likewise. The inequality of the sacs is probably due to the fact that those used were of a commercial variety, as indicated below. To continue:

Further, we have made two experiments on animals, one with diphtheria toxin, the other with an alkaloid which it may be assumed must readily traverse the membrane—strychnine.

Experiment with diphtheria toxin: A guinea pig (600 g.) received in its peritoneal cavity a collodion sac (commercial) inclosing 2 c.c. of broth containing 5 g. of diphtheria toxin, fatal at 0.05 g. for a 250 g. guinea pig. It survived. Another (500 g.) received an intraperitoneal injection of 0.25 g. (a dose one-half less than that of the preceding) of the same toxin. It died in less than forty hours.

A dose of diphtheria toxin, much greater than the fatal dose, is thus perfectly tolerated in a collodion sac.

Experiment with strychnine: A guinea pig (540 g.) received July 7 in the peritoneal cavity a collodion sac (commercial) with 0.75 c.c. of an aqueous solution containing ¾ mg. of sulphate of strychnine. The following day it showed nothing in particular; no contractions. It died on the 14th, but its death was not due to strychnine. The operation wound had been opened, and allowed the intestine to protrude; the body was supple; on the evening of the 13th there had been no contraction. The contents of the sac, withdrawn and injected subcutaneously into another guinea pig, did not cause death.

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Consequently the toxin diffused out of the sac, but so gradually as to be tolerated.

. . . . We are convinced that the collodion wall is far from being as permeable to dissolved substances as bacteriologists have admitted up to the present, and as a consequence the different interpretations of experiments made on this principle deserve to be held in doubt.

These experiments are of interest in that they call attention to the imperfect permeability of the collodion wall—a fact which our own experiments will further substantiate. We are inclined to believe, however, that with sufficiently thin sacs the dialysis in the above experiments would have been more marked.

We wish further to call attention to the experiments of Crendiroupoulis and Ruffer,[14] who find the toxin of pyocyaneus is partially dialyzable through the collodion wall, but not through walls of too great thickness. They state further that the comparison of the toxin filtered through the bougie with the toxin dialyzed through collodion shows a difference in pathogenic energy quite in favor of the former. They have, however, been able to produce with dialyzed toxin all the symptoms of pyocyaneus intoxication without exception, provided the quantity injected be sufficiently large.

The sacs employed in the following experiments were made according to the method described by Gorsline (*loc. cit.*). They were about 2½ inches in length and ½ inch in diameter, and were made by turning the tube used in making the sacs twice in dilute collodion, producing a sac of extreme thinness. These sacs were free from air bubbles, opaque areas, or flaws of any nature, were practically invisible when immersed in water, and collapsed immediately on emptying. Each sac was tested for possible leaks by immersing while empty in distilled water and distending with air under pressure. Those sacs showing evidences of leakage were discarded.

EXPERIMENT X.

Seven thin collodion sacs were prepared as above described. Each was mounted on a glass tube, some three inches in length, made by cutting off the end of a small test-tube. The sac was slipped over the end of this tube for a distance of a half-inch, and then contracted on the glass by rotation over a narrow flame, as described by Novy.[15] This joint was finally recoated

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with collodion. As stated above, each sac was tested for leaks before it was used.

A quantity of saliva was filtered through a single thickness of ordinary filter paper. In each of six sacs, labeled 1–6 inclusive, were placed 5 c.c. of this saliva. In the control sac, marked *C*, were placed 5 c.c. of same saliva, previously boiled for five minutes to destroy the enzyme. Each sac was immersed in 20 c.c. (Sac 5 in 25 c.c.) of a 0.01 per cent. starch solution, contained in a test-tube on foot. In no sac did the liquid reach the joint, thereby insuring impossibility of capillary escape of liquid from sac.

The tubes containing these sacs were set aside in an incubator at 37°. At the end of three hours 2 c.c. of the liquid were withdrawn from each tube and tested with several drops of dilute iodine with the following results:

TABLE XVII.

Contents of Tube	Reaction Produced by Iodine
C	Blue
1	Achromic
2	Achromic
3	Achromic
4	Achromic
5	Pink
6	Achromic

The contents of Tube 5 and of the control tube were again tested at the end of the fourth hour; the latter gave a blue, starch reaction, while the former was achromic.

The sacs were again tested and found to be free from leaks. The only way by which the inversion of the starch could have been brought about was by the dialysis of the ptyalin through the collodion membrane.

Further similar experiments gave identically the same results and clearly demonstrated that ptyalin will dialyze through the collodion membrane.

The next point was to determine whether or not ptyalin could be filtered through such a membrane. For this purpose the following experiment was made:

EXPERIMENT XI.

A thin collodion sac was attached, in the manner above described, to a glass tube passing through a rubber stopper. The joint was further strengthened by winding with a silk thread, and again recoating with collodion. The sac was of the same thickness as those used in the above dialysis experiments. The empty sac was tested under water by forcing air into it at 3 inches' pressure, and was found to be free from leaks. The sac thus mounted was placed in a test-tube on foot, provided with a side arm. The rubber stopper and the mouth of the tube formed an air-tight joint. Five cubic centimeters of the undiluted saliva, previously filtered through a single thickness of ordinary filter paper, were then introduced into the sac. The side arm of tube was connected with a gently working aspirator, and a suction pressure of 2½ inches was maintained for one and one-half hours. During this time droplets of perspiration appeared on the outer side of the distended sac, slowly

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became confluent, collected in drops at the lower end of the sac, and fell to the bottom of the test-tube. In this way 1.5 c.c. of the contents of the sac were drawn through the wall and collected at the bottom of the test-tube. The filtering liquid and the control were protected during the process against the action of light by covering with black cloth.

One cubic centimeter of this filtrate was added to 30 c.c. of a 1 per cent. starch solution. An equal quantity of the control or paper-filtered saliva was added to a similar amount of starch solution. A third tube, containing 30 c.c. of the starch solution, served as a blank control. These tubes were set aside at 37–38°, and portions of the liquid were removed, from time to time, from each tube and tested with iodine. The result was as follows:

1 c.c. control + 30 c.c. starch solution — achromic in 20 min.
1 c.c. filtrate + " " " — blue in 22 hrs.
Starch control — blue in 22 hrs.

This experiment shows that the ptyalin was completely retained by the collodion filter.

The filter sac employed above was well washed, and then 5 c.c. of fresh filtered saliva were introduced. It was then immersed in 20 c.c. of 0.01 per cent. starch solution and placed in an incubator at 37°. The experiment was controlled with a sac similarly immersed containing 5 c.c. of boiled saliva. In a control test with iodine at the beginning of the dialysis the starch gave a deep blue color. At the end of three hours the starch in both tubes showed complete inversion. The fact that the control or heated saliva behaved thus showed that the boiling was insufficient to destroy its activity.

The sacs were again tested and found free from leaks. Thus, the same sac which refused passage to ptyalin, when used as a filter, allowed the enzyme to pass through it readily by dialysis. This fact is further substantiated by the following similar experiment:

Some saliva was placed in a new thin collodion sac and aspirated as before. Three and two-fifths c.c. of a water-clear liquid filtered through in one and one-half hours at a pressure varying from 2¾ to 3¼ inches. This filtrate was tested as before at 38°.

1 c.c. of control saliva + 30 c.c. of 1 per cent. starch solution—achromic in 30 min.
1 c.c. saliva filtrate + 30 c.c. 1 per cent. starch solution—blue in 45 hrs.
Blank control (30 c.c. of starch solution) blue in 45 hrs.

The control was made with the unfiltered saliva remaining in the sac. Therefore the activity of the ptyalin was not destroyed by the action of the collodion wall or by the pressure. The sac was tested before and after use at a pressure of 3¼ inches, and was found to be free from leaks. It was then employed for a dialysis test, for which purpose 5 c.c. of fresh saliva were introduced into the sac, which was then immersed in a test-tube on foot containing 20 c.c. of a 0.01 per cent. starch solution. A control sac similarly immersed, containing 5 c.c of saliva previously boiled for five minutes, was also set aside. The tubes were placed in the incubator at 37°. An initial control test with iodine gave a deep-blue color. At the end of one hour and at the end of one hour and forty minutes portions of starch were removed from each tube and tested with iodine with the following results:

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TABLE XVIII.

	Control	1 Hour	1 Hr. 40 Min.
Used sac	Blue	Pink	Achromic
Control sac	Blue	Blue	Blue

The foregoing experiments demonstrate the ready dialysis of ptyalin through the collodion sac, and the perfect impermeability of the latter to ptyalin when used as a filter. This impermeability is not due to fixation, for if the collodion wall could fix ptyalin, dialysis could not occur.

The fact that ptyalin is retained from saliva on passage of the latter through hardened filter paper; that it is not retained by the Berkefeld bougie; that it is not affected by aeration; that paper bits which have been immersed in saliva, when washed entirely free from saliva, will yet invert starch; that this paper will not give up this property on washing with water, nor yield to the water any diastatic property, and that it yields its retained ptyalin readily to glycerin, goes to show that pytalin is *fixed* by hardened filter paper.

TAKA DIASTASE.

FILTRATION THROUGH PAPER.

The solutions of taka diastase (Parke, Davis & Co.), of a dilution of 1:4,000 were first filtered through a single thickness of ordinary filter paper. They were then subjected to filtration through eight double filters of hardened filter paper, and each filtrate was tested in the manner as given under ptyalin. In the several filtrations tried the "8 × 2" filtrate showed itself but little, if indeed at all, weaker in activity than the original control. Inasmuch as this 1:4,000 solution was but half as strong as a 10 per cent. solution of ordinary saliva, it was not deemed necessary to work with greater dilutions.

Experiments in the aeration tube likewise failed to demonstrate any fixation, although the activity of the enzyme was decreased by the aeration. This weakening was due either to the aeration itself, or to the surface action of the paper and the beads over which it was aerated, or to both.

The enzyme passed through the Berkefeld filter at vacuum pressure as readily as it passed through the "8 × 2" filters.

DIALYSIS.

The following, one of several similar experiments, indicates the dialysability of taka diastase:

Four thin collodion sacs were prepared and mounted as previously described, and labeled *C*, 1, 2, and 3. In each of 1, 2, and 3 were placed 5 c.c.

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of a 1:1,000 distilled water solution of taka diastase, previously filtered through a single thickness of ordinary filter paper. In *C* were placed 5 c.c. of the same solution, previously boiled for five minutes. This served as control solution. Each sac was immersed in a test-tube on foot containing 20 c.c. of a 0.01 per cent. starch solution, and the whole was then placed in the incubator at 36° for the purpose of dialysis. A control test of the starch before immersion of the sacs gave a deep-blue coloration on addition of iodine. Portions of 2 c.c. were removed from each of the test-tubes at the end of three, six, nine, and twenty-four hours, and were tested with dilute iodine to observe progress of digestion, if any. The results obtained are given in the following table:

TABLE XIX.

Time	Tube *C*	Tube 1	Tube 2	Tube 3
3 hrs.	Blue	Blue	Blue	Blue
6 hrs.	Blue	Light blue	Light blue	Light blue
9 hrs.	Blue	Pink	Pink	Pink
24 hrs.	Pink	Achromic	Achromic	Achromic

The sacs were tested before and after dialysis, and were found free from leaks.

Taka diastase therefore dialyzes, though slowly, through the collodion sac.

FILTRATION THROUGH THE COLLODION SAC.

The following table gives the results of several filtrations of taka diastase through thin collodion sacs, under conditions similar to those given in connection with ptyalin. The taka diastase was employed in a 1:1,000 solution in 0.7 per cent. NaCl. The solution was passed through a single thickness of ordinary filter paper before collodion filtration. Controls were made in each instance from the unfiltered liquid remaining in sac after filtration. The controls and filtrates were tested by adding 1 c.c. of the liquid to 30 c.c. of a 1 per cent. starch solution and digesting in water bath at 37°. A blank control was made in each instance with 30 c.c. of the starch solution. The sacs were tested at a pressure of three inches and were found to be free from leaks before filtration.

TABLE XX.

No.	Filt. Pressure	Time of Filt.	Am't Filtered	Color Reaction with Iodine of		
				Starch Control	Diastase Control	Filtrate
	(Ins.)	(Hrs.)	(c.c.)			
1.....	2½-3½	2	3.8	Blue in 19 hrs.	Achr. in 2-2½ hrs.	Purple in 6 hrs. Achr. in 19 hrs.
2.....	2-3	2	4.2	Blue in 17 hrs.	Achr. in *ca.* 3 hrs.	Purple in 3½ hrs. Achr. in 17 hrs.
3.....	2-3½	1½	3.5	Blue in 24 hrs.	Achr. in 1 hr. 40 min. -2 hrs. 50 min.	Achr. in 22-24 hrs.
4.....	3⅛	1½	5.0	Blue in 24 hrs.	Achr. in *ca.* 3 hrs.	Achr. in 18-24 hrs.

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The sacs used in filtrations 2, 3, and 4 were tested after filtration and found to be free from leakage. The sac used in filtration 1 showed leakage at 2½ inches.

The sac used in filtration of No 2, after thorough washing, was filled with 5 c.c. of the 1 : 1,000 diastase solution and immersed in 20 c.c. of a 0.01 per cent. starch solution. In another sac were placed 5 c.c. of the same solution, previously boiled, and this was immersed similarly, and served as a control. Both sacs were placed in the incubator at 37°. A control or initial test of the starch solution gave a blue reaction with iodine. At the end of one and one-half hours 2 c.c. of the starch solution were removed from each tube and tested with iodine. The starch in the control tube gave a deep-blue color, whereas that in the tube containing sac No. 2 gave an achromic reaction.

The sac, after the dialysis, was tested at 3 inches' pressure and was found to be free from leakage.

It will be seen from the experiment that, although the sac greatly retarded the passage of taka diastase, by filtration, it permitted a ready dialysis of the enzyme.

It has been shown that ptyalin is totally retained by the collodion filter, but taka diastase passes through to a considerable extent. It seemed possible that the retention of ptyalin was due to the presence of mucin, and if such was the case, a similar behavior could be expected of taka diastase under like conditions. The following experiments were made in order to show the effect of mucin:

A quantity of saliva which had been filtered through a single thickness of ordinary filter paper was boiled for five minutes in order to destroy the ptyalin. After cooling, 10 c.c. of this saliva were added to 10 c.c. of a 1 per cent. taka diastase solution and thoroughly mixed. The mixture was filtered through a collodion sac in the manner previously described. The filtrate and the control were tested by adding 1 c.c. of the liquid to 20 c.c. of the 1 per cent. starch solution. Blank controls were made as before. The following table gives the results of two such filtrations:

TABLE XXI.

No.	Filt. Pressure	Time of Filt.	Am't. Filtered	Iodine Reaction of		
				Starch Control	Diastase Control	Filtrate
	(Ins.)	(Hrs.)	(c.c.)			
1.....	3-3¼	1¼	3.3	Blue in 20¼ h.	Achr. in 6 hrs. +	Purple in 6 hrs. / Achr. in 20¼ hrs.
2.....	3¼	1¼	3.8	Blue in 20 hrs.	Purple in 3 hrs. / Achr. in 17½ hrs.	Deep purple in 3 hrs. / Red in 20 hrs.

At the close of the experiments the sacs were tested at a pressure of 3¾ and 5 inches respectively, and were found to be free from leakage.

The preceding filtrations were with mixtures of boiled saliva and taka

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diastase. The two following experiments were made with raw saliva and 1 per cent. taka diastase in equal parts. Inasmuch as ptyalin does not filter through the collodion sac, a positive result must be due to the passage of taka diastase, notwithstanding the presence of mucin and other saliva constituents.

EXPERIMENT I.

Three cubic centimeters of the mixture filtered through a sac in 1 hour at a pressure of 2½–3½ inches. The sac was tested after filtration and showed no leak at 5 + inches.

Two cubic centimeters of this filtrate added to 20 c.c. of a 1 per cent. starch solution and left at 36° gave a purple test in 5 hours and an achromic reaction in 21 hours.

The blank control (20 c.c. of a 1 per cent. starch solution left at 36°) gave a blue reaction at the end of 21 hours.

The activity of the mixture in the sac was tested by adding 2 c.c. to 20 c.c. of a 1 per cent. starch solution and left at 36°. An achromic reaction was obtained in 7 minutes.

EXPERIMENT II.

Three cubic centimeters of the mixture filtered through a sac in 1 hour at a pressure of 2¼–3¼ inches. No leak could be detected in the sac after filtration, even at 6 inches' pressure.

Two cubic centimeters of this filtrate were added to 20 c.c. of the starch solution and left at 36°. An achromic reaction was obtained in 32 to 42 minutes.

The blank control (20 c.c. starch solution at 36°) gave a deep purple at the end of 42 minutes.

Two cubic centimeters of the mixture remaining in the sac were added to 20 c.c. of the starch solution at 36°. Iodine gave an achromic reaction in 40 minutes.

These experiments show conclusively that the presence of mucin does not prevent the passage of taka diastase through the collodion filter. It is probably also without effect on the ptyalin molecule.

Summing up, it will be seen that taka diastase is not removed from its solution by filtration through paper; it passes readily through the Berkefield filter, partially through the collodion filter, and dialyzes through the collodion membrane. It is injuriously affected by aeration.

RENNET.

EXPERIMENT I.

Paper filtration.—That rennet is removed from its solution by filtration through hardened filter paper is evidenced by the following results:

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The rennet solution used in this experiment was prepared by dissolving one tablet of the commercial preparation (Chr. Hansen) in 100 c.c. of physiological salt (0.7 per cent.) solution; the resulting solution was then filtered through a single thickness of ordinary filter paper. This clear solution was not used directly, but served for the preparation of a 1:40,000 dilution. In each experiment 200 c.c. of the 1:40,000 rennet solution, in physiological salt, were used. Of this amount 195 c.c. served for the purpose of filtration, and 5 c.c. for the control tests. The rennet activity of each filtrate was tested by adding 1 c.c. of each to 5 c.c. of fresh milk in a test-tube kept at about 37° in a water-bath and observing the time required for coagulation. The rennet experiments covered a number of days, and fresh dairy milk was obtained each morning for that day's work. The filtering liquids as well as the controls were kept under black cloth. A common control was used for the "6×2" and "8×2" filtrates. The filter paper employed was ordinary hardened filter paper cut into squares of 15 cm. each.

TABLE XXII.

1 C.C. OF FILTERED 1:40,000 RENNET SOLUTION + 5 C.C. MILK AT 36°.

No.	DURATION OF FILTRATION	DIGESTION TEMP.	TIME REQUIRED TO PRODUCE COAGULATION BY						
			"2×2"		"4×2"		"6×2"		"8×2"
			Control	Filtrate	Control	Filtrate	Control	Filtrate	Filtrate
	Min.		Min.	Min.	Min.	Min.	Min.		
1...	110	36°	58	85–135	45–55	238	52	5 hrs. 15 min.	Neg. 18 hrs.
2...	130	34–37°	26–32	52	27	94–123	25	Slight Coag. 16 hrs.	Slight Coag, 16 hrs.
3...	170	37°	90	152–197	94–109	110–155	73–103	5–6 hrs	5–16 hrs.

The experiments tabulated above clearly show that successive filtrations of a rennet solution rapidly decrease its activity. In order to determine whether or not the removal of the enzyme was due to fixation, aeration over crumpled pieces of filter paper was resorted to as in the case of the ptyalin experiments already described.

EXPERIMENT II.

Effect of aeration.— In these experiments the liquid was aerated in the special tube mentioned heretofore, over bits of filter-paper, glass beads, and garnets. It was found that after three hours' aeration, either over crumpled paper bits or over glass beads, the liquid within the tube was without any coagulating action. The fact that the glass beads exerted the same action as the bits of paper indicated that the enzyme was destroyed and not merely fixed or retained by the paper. Moreover, all attempts at extraction with glycerin of enzyme possibly fixed by the paper bits produced negative results.

In order to make sure that the rapid destruction of the enzyme was not due to the alkalinity resulting from the action of the water on the walls of the tubes, on the beads themselves, or on

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the plug of glass-wool employed in keeping the paper bits in the tubes, the experiments were repeated with aeration tubes of Bohemian glass. One of these was filled with thoroughly cleansed garnets in place of the beads and the glass-wool. A second tube was filled with crumpled bits of paper, while as a further check, a third aeration tube was set up as a control in which the liquid was aerated alone, that is, not over garnets or paper.

Under these conditions the same ready destruction of the enzyme was obtained. In the control aeration tube the destruction was less rapid, six or more hours being required to destroy totally the enzyme, whereas over paper or garnets the rennet was destroyed in two to three hours. Control tests made with the unaerated rennet solution showed unimpaired activity during the time of these experiments. Moreover, it should be stated that tests were made with phenolphthalein of all liquids after aeration and showed an absence of alkalinity.

These facts then indicate that rennet in solution is readily destroyed by aeration, which destruction is hastened by the surface action of paper bits or garnets.

Effect of Berkefeld filters.—The retention of enzymes by porous porcelain filters has been observed in numerous instances. Gautier[16] called attention to the fact that, in the filtration of gastric juice through the porcelain bougie the pepsin was largely retained on the filter. Fermi and Pernossi[17] later experimented on the passage of trypsin and pepsin through the Chamberland bougie. They filtered a 1:500 solution of trypsin, containing five per cent. NaCl, through the bougie at a pressure of two atmospheres, and found a decrease of about one-half in the activity of the filtrate as compared with that of the original solution. The filtration of a 1:200 solution, containing no NaCl, caused a loss of about one-fourth of its strength, while the filtration of a 1:200 solution containing two per cent. of Na_2CO_3 resulted in the loss of about half of the strength of the solution.

In order to show that the decrease in activity was not due to the removal of undissolved trypsin which might be present, they filtered a 1:200 solution of the enzyme through the bougie at one atmosphere pressure. This filtrate was again filtered through

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the bougie, and the resulting filtrate was passed through the filter once more, giving thus a triple filtration of the solution. They tested their controls and the filtrates by addition to gelatin, and found that the successive filtrates progressively decreased in activity. This fact was taken to prove that the weakening of the filtrate was not due to the retention of undissolved particles.

Setting aside the possibility that the trypsin could be destroyed by the filtration process, they reached the conclusion that the loss of the activity of the filtered solution was due to the retention of the molecules by the filter. To quote their words:

"We come to the conclusion that trypsin passes through the porcelain filter in such a way that, when one repeats (four to five times) the filtration of the same solution, the trypsin remains entirely on the filter."

They also observed a weakening in the activity of solutions of pepsin by refiltering through the porcelain bougie.

Achalme[18] also noted the retention of pancreatin by the bougie, but according to his statement the pancreatin loses only about 25–35 per cent. of its diastatic power, when filtered at a temperature of 35–38°.

Lea (*loc. cit.*) observed the retention of the urea ferment by the porous battery cell. Miquel (*loc. cit.*) was for a long time unable to pass urase through the porcelain bougie, but finally succeeded in obtaining a passage of the ferment after displacing the atmospheric air in the pores of the filter with illuminating gas.

Fermi[19] observed the total retention by the porcelain filter of the inverting enzyme of B. megaterium.

Buchner[20] succeeded in obtaining a partial passage of zymase through both the Berkefeld and Chamberland bougies. Fernbach[21] has shown that the sucrase of Aspergillus niger is totally retained by the porcelain bougie, whereas the sucrases of the Champagne yeast, of Saccharomyces Pastorianus, and of pale ale pass *en totalité* through the filter. According to Effront,[22] the invertin of yeast passes the Chamberland filter, whereas that of aspergillus does not. Ehrlich and Morgenroth[23] have pointed out that the complement in goat's blood causing the solution of rabbit corpuscles is retained on the Pukallfilter.

Vedder,[24] in working on the bacteriolytic complements in the

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rabbit's blood, filtered the serum through Chamberland and Berkefeld filters, with the following results:

The complements for all the organisms tested (B. typhosus, B. coli communis, B. dysenteriæ, and S. pyogenes aureus) may be separated from the serum by filtration through a Chamberland filter; but by passage through a Berkefeld filter the complements for certain organisms are retained (B. coli communis, S. pyog. aureus, and B. dysenteriæ, partially), while those for others (B. typhosus, B. dysenteriæ partially) pass through — a further indication of the multiplicity of the bacteriolytic complement. By filtration through a Chamberland filter it is possible to separate the agglutinins from the bactericidal substances of a serum.

We have known but little hitherto about the filtration of rennet. According to Oppenheimer,[25] Lea[26] found that vegetable as well as animal rennet was retained by the kaolin filter. In the reference cited by Oppenheimer we find no authority for such a statement. Lea found a milk-coagulating ferment in the plant Withania coagulans. In order to decolorize its solution he added finely divided kaolin, which addition resulted in the weakening of the enzyme. No mention is made of filtration through kaolin. In view of a more recent paper by Lea (and Dickinson),[27] in reply to Fick's theory as to the action of rennet, it seems improbable that he has witnessed any such retention. We quote the following experiments:

Some milk was placed in a narrow (2 cm. wide) porous battery cell made of extremely thin earthenware. The cell was then immersed in a beaker of rennin solution, so that the level was the same inside and outside the cell, and digested at 40° for seventeen hours. At the end of this time there was not a trace of clot in the milk or on the inner surface of the cell. The experiment was repeated for a period of twenty-seven hours, with the same result. It appeared to us that if Fick's view is correct, then a clot might have been expected to form in the above experiment, if not throughout the whole mass of the milk, at least along the inner walls of the cell; but no trace of a clot was observed. It would perhaps, however, be unwise to lay too much stress on this experiment, for we know but little of what is happening in the pores of the cell's wall when it is used as a septum between two different fluids. But it is scarcely conceivable that the rennin should not have come in contact with the milk, bearing in mind that such a cell is permeated by pores through which a filtration of fluids can be carried on under pressure.

Lea and Dickinson did not attempt, however, to filter rennet solution through these pores under pressure.

Roberts[28] (1880) found that the brine extract of pancreas

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when filtered under vacuum pressure through porous earthenware possessed an undiminished faculty for curdling milk, but it had almost no power of dissolving the curds. The curdling ferment had evidently traversed the earthenware freely, but only traces of trypsin had passed through.

Conn[29] (1892) separated rennet from the proteolytic enzyme of certain bacilli (B. mesentericus vulgatus?) by mixing the products of the organisms with milk and allowing coagulation to occur. This coagulum was broken up, then well shaken with distilled water, and the resulting solution filtered through porcelain. H_2SO_4 was added until the liquid contained 0.1 per cent., and NaCl was then added to saturation. A white granular-looking scum floated to the top, which was found to be almost pure rennet. The proteolytic enzyme remained behind in the brine.

Korschun[30] recently published a paper from which it appears that he has filtered rennet solution through both Berkefeld and Chamberland bougies. He gives no description of his process except that he used a little Berkefeld bougie. He finds a partial, but not complete, retention of the coagulating enzyme by the filter.

We know but little of the filter employed by Roberts. If, however, his results are correct, they give us an important distinction between pancreatic and gastric rennet; the passage of the rennet employed by Conn may be due to the fact that it is of vegetable origin. As regards Korschun's work, we are inclined to believe that he used imperfect bougies, or a rennet differing in properties from that employed by us. Our own experiments will show that the Berkefeld bougie, in perfect condition, will deny passage to the enzyme rennet, whether the filtration force be simply gravity, or 100 pounds' pressure to the square inch. On the other hand, old used bougies become more or less permeable.

EXPERIMENT III.

In this, as in all succeeding experiments, the rennet solution was filtered, either in the dilute form in which it was used, or previous to dilution, through one thickness of ordinary thin filter paper to insure absence of undissolved particles of rennet. The rennet was either that of Chr. Hansen or of Parke, Davis & Co., which commercial preparations were found to be identical in strength and behavior. Seven-tenths per cent. NaCl was used as the solvent for these solutions. The bougies were sterilized in every instance before each

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individual filtration. It was observed that new Berkefeld bougies contain a considerable quantity of alkali, which passes into solution in the liquids traversing the bougie. Hence, before each individual filtration the bougie was washed by drawing through it hot distilled water, until the wash-water failed to give an alkaline reaction to phenolphthalein. The bougie was then cooled down to room temperature by passage of cold distilled water. Every enzyme filtrate was tested and found to be alkali-free. In the high-pressure filtration experiments the bougies were further washed by forcing distilled water through them at the pressure employed in the subsequent rennet filtration. The filtering apparatus employed was that described by Novy.[21]

Filtration by gravity.—One hundred and eighty cubic centimeters of a 1:10,000 rennet solution filtered through a Berkefeld bougie at gravity pressure in two hours. A portion of the solution was previously set aside to serve as control. The filtrate and control were tested by adding 1 c.c. of each to portions of 5 c.c. of milk. Blank controls of milk were made to indicate coagulating action, if any, of micro-organisms. The digestion temperature was 36°.

The tests which were made in this experiment resulted as follows:

1 c.c. of control enzyme + 5 c.c. milk gave coagulation in 23 min.
1 c.c. filtrate + 5 c.c. milk gave no coagulation in 6 hrs.
Milk control gave no coagulation in 6 hrs.

It appears, therefore, that Rennet does not pass through the Berkefeld bougie in gravity filtration.

EXPERIMENT IV.

Filtration at vacuum pressure. — The following tabulated set of filtrations was carried on by aspiration. In the first three filtrations the liquid was allowed to pass through the bougie immediately upon beginning of suction, the pressure increasing during the filtration process to the point indicated in the table. In the latter three filtrations the receiving flask was exhausted before admission of the liquid, so that the entire filtration was carried on at the pressure indicated in the table. Milk controls indicated in the table are blank controls consisting of merely 5 c.c. of milk, without addition of enzyme. The tests of controls and filtrates were made by adding 1 c.c. of the liquid tested to 5 c.c. of milk.

TABLE XXIII.

No.	Dilution	Pressure	Duration of Filtration	Amt. Filt.	Temp. of Digestion	Coagulating Action of		
						Control		Filtrate
						Milk	Rennet	
		Ins.	Min. Sec.	c.c.				
1.....	1:20,000	26⅞	1 50	195	37.5–36°	.....	Coag., 40–44 m.	Neg. in 20 hrs.
2.....	1:10,000	27¼	1 30	195	40°5	.....	Coag. in 20 m.	Neg. in 8 hrs.
3.....	1:10,000	27½	0 62	196	39°	.....	Coag. in 21 m.	Neg. in 2 hrs.
4.....	1:10,000	27⅛	1 30	195	36°	Neg., 2 h. 5 m.	Coag. in 19 m.	Neg., 2 h. 5 m.
5.....	1:10,000	26½	0 75	195	35°	Neg. in 11 hrs.	Coag. in 21 m.	Neg. in 11 hrs.
6.....	1:10,000	27⅝	0 85	195	35°	Neg. in 7 hrs.	Coag. in 23 m.	Neg. in 7 hrs.

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As above mentioned, Achalme found that pancreatin loses only 25–35 per cent. of its activity when filtered through the porcelain bougie, provided the solution is heated to 35–38°. In the following filtrations not only were the solutions warmed to the temperature indicated in the table, but the filters were also heated to the same temperature, respectively, by the repeated passage of warmed distilled water through the bougie. Thus, in the following filtrations the solutions of 37, 43, and 46° passed through filters of the same respective temperatures. To determine the effect, if any, on the enzyme of warming, controls were made with the liquid both at room temperature and at filtration temperature.

TABLE XXIV.

No.	Dilution	Pressure	Time of Filtration	Amt. Filt.	Temp. of Filtration	Temp. of Digestion	Coagulating Action of		
							Control		Filtrate
							Room Temp.	Filt. Temp.	
		in.	sec.	c.c.					
1....	1 : 10,000	28	65	190	37°	37–35°	Coag. in 21 min.	Coag. in 21 min.	Negative in 3 hrs. 50 min.
2....	1 : 10,000	27¾	....	190	43	39–37	20 min.	20 min.	16 hrs.
3....	1 : 20,000	26¾	110	190	46	37	46 min.	46 min.	4 hrs. 30 min.

An attempt made to filter at 52° with its corresponding increase in molecular motility, however, resulted in the destruction of the enzyme. In the four filtrations the liquids were gently warmed over the Bunsen flame, and then filtered immediately upon arriving at the desired temperature.

We have already called attention to the fact that Miquel succeeded in filtering urase through the porcelain bougie in an atmosphere of illuminating gas, whereas the enzyme failed to traverse the bougie in the presence of air. The following experiment is designed to demonstrate the behavior of rennet as regards filtration in an atmosphere of inert gas:

a) One hundred and ninety-five cubic centimeters of 1:10,000 rennet solution were filtered through the Berkefeld bougie at 28 inches' pressure in 45 seconds in an atmosphere of air. Controls and filtrates were tested as before.

1 c.c. control + 5 c.c. milk at 36°—coagulated in 20 min.
1 c.c. filtrate + 5 c.c. milk at 36°—coagulated in about 5 hrs.
Milk control negative in 5 hrs.

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b) Scrubbed and sterilized the bougie used in *a*. Into the mouth of the filter globe inserted a rubber stopper with two apertures, one of which carried a tube connected with the illuminating-gas supply, the other carrying the tube of a separatory funnel. Closed the cock of the funnel and poured into the latter 190 c.c. of 1:10,000 rennet solution. Exhaustion was produced in the globe, bougie, and receiver, and gas was then admitted into the apparatus until the pressure fell to about five inches. Exhaustion was again produced, and gas was readmitted until the pressure fell to about five inches. The rubber connection between the receiver and the cylinder was then clamped, and the receiver exhausted, gas in the meantime continuously passing into the globe and bougie. When the receiver was exhausted, the clamp was released, and at the same time the enzyme solution was allowed to pass from the separatory funnel into the globe, leaving behind enough liquid in the funnel, however, to prevent entrance of air into the globe. This remaining liquid was also used for the control test. Liquid thus passed through the bougie in an atmosphere of illuminating gas, and in the absence of air.

Pressure, 27⅞ inches; time of filtration, 60 seconds; amount filtered, 185 c.c.

1 c.c. control + 5 c.c. milk at 36 degrees—coagulated in 23 min.
1 c.c. filtrate + 5 c.c. milk at 36 degrees—coagulated in 6–7 hrs.
Milk control—coagulated in 6–7 hrs.

c) Scrubbed and sterilized the bougie and washed the same thoroughly Then filtered 195 c.c. of 1:10,000 rennet solution in an atmosphere of air at a pressure of 28⅛ inches in sixty seconds.

1 c.c. control + 5 c.c. milk 36°—coagulated in 15 min.
1 c.c. filtrate + 5 c.c. milk 36°—coagulated in 6–7 hrs.
Milk control—coagulated in 6–7 hrs.

It does not seem, after comparison with filtrations *b* and *c*, that any of the enzyme passed through the bougie in filtration *a*. The milk used in this experiment was undoubtedly very rich in organisms, and had we kept the milk control under observation a few minutes longer, we should no doubt have observed coagulation. As before mentioned, milk was obtained fresh each morning for the work of the ensuing day, but it seems probable that on this particular day an aged milk was substituted.

Rennet, therefore, does not pass through the Berkefeld bougie in an atmosphere of illuminating gas. Is gas absolutely inert toward rennet, or is the absence of activity in the filtrate due to the destruction of the enzyme by the gas? The following experiment was made in order to answer this question:

In each of three test-tubes of Bohemian glass, each provided with a side arm, were placed 20 c.c. of a 1:10,000 rennet solution. The tubes were designated *A*, *B*, and *C*, respectively.

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Through rennet in *A* gas was passed for five minutes; the rennet in *B* was aerated for five minutes, entrance of foreign particles into the tube being guarded against by a cotton plug in the side arm; the contents of *C* served as control. Each of these was tested, with the following results:

1 c.c. of control + 5 c.c. milk at 36°—coagulated in 23 min.
1 c.c. of *A* + 5 c.c. milk at 36°—coagulated in 21 min.
1 c.c. of *B* + 5 c.c. milk at 36°—coagulated in 21 min.
Milk control was negative.

Illuminating gas can therefore be said to be without effect on rennet solutions.

The foregoing experiments show that rennet solutions do not pass through the Berkefeld bougie at vacuum pressure, either at room temperature or at higher temperature, or in an atmosphere of illuminating gas.

EXPERIMENT V.

Filtration at fifty pounds' pressure.—The procedure of pressure filtration employed in the following experiment is that described by Novy (*loc. cit.*). Save in the first two filtrations, all filtered liquids were forced directly into the spherical receiver described in the text, thereby obviating the effect on the filtrate of possible leakage in the apparatus.

A. One-hundred and ninety-five cubic centimeters of a 1:10,000 rennet solution were subjected to filtration at 50 pounds' pressure through the Berkefeld bougie. The time of passage was about fifteen seconds. The tests of control and filtrate resulted as follows:

1 c.c. control + 5 c.c. milk at 34–35°—coagulated in 18 min.
1 c.c. filtrate + 5 c.c. milk at 34–35°—coagulated in 9–11½ hrs.

No milk control was made, but it is evident that no enzyme passed through the bougie.

The bougie was employed in the meantime in a filtration test at 100 pounds' pressure (Experiment VI*a*), after which it was used for another filtration at 50 pounds. One hundred and ninety-five cubic centimeters of the solution were filtered in about ten seconds.

1 c.c. control + 5 c.c. milk at 36°—coagulated in 23 min.
1 c.c. filtrate + 5 c.c. milk at 36°—coagulated in 40 min.
No milk control.

In the first filtration the bougie had previously been used for nothing but vacuum-pressure filtration, and no enzyme passed through the bougie at 50 pounds' pressure. An ensuing filtration at still higher pressure had evidently worn channels in the filter, and a second filtration at 50 pounds caused a retention of less than half of the enzyme.

B. In this test a new bougie was employed. One hundred and ninety cubic centimeters of a 1:10,000 rennet solution were filtered in eight seconds at 50 pounds' pressure.

1 c.c. control + 5 c.c. milk at 36°—coagulated in 24 min.
1 c.c. filtrate + 5 c.c. milk at 36°—negative in 2 hrs. 15 min.
Milk control—negative in 2 hrs. 15 min.

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Observations not taken after two and a quarter hours.

In the meantime the bougie was used for four filtrations at 100 pounds' pressure. Then another filtration was made at 50 pounds. One hundred and ninety-five c.c. were filtered in ten seconds. The tests resulted as follows:

1 c.c. control + 5 c.c. milk at 35° — coagulated in 21 min.
1 c.c. filtrate + 5 c.c. milk at 35° — coagulated in 32 min.
The milk control was negative.

One hundred and ninety-five cubic centimeters of a 1:10,000 rennet solution, made up from the same 1:1,000 solution as that used in the preceding filtration, were filtered through another bougie (not new) at 50 pounds' pressure in ten seconds:

1 c.c. control + 5 c.c. milk at 34.5° — coagulated in 22 min.
1 c.c. filtrate + 5 c.c. milk at 34.5° — coagulated in 2 hrs. 20 min.
The milk control was negative.

The preceding experiments go to show that repeated filtrations under high pressure through the same bougie cause a decrease in the retaining properties of the bougie. The following series of filtrations was designed to bring out this point more fully.

C. Six filtrations of a 1:10,000 rennet solution were made through the same bougie at 50 pounds' pressure. The bougie was sterilized before each filtration by heating in an autoclave in about two liters of distilled water to 136–140° C. Twelve hundred and twenty cubic centimeters of a 1:10,000 rennet solution were made up and filtered in the six portions indicated below. The tests of filtrates and control were made as in the preceding experiments. Milk controls, without the addition of rennet, were also made as before. The digestion temperature was 35°.

TABLE XXV.

No.	Amount of Filtrates	Time of Filtration	Coagulating Action of		
			Control		Filtrate
			Milk	Rennet	
1.....	195 c.c.	9 sec.	Neg.	Coag. in 20 min.	Coag. in 1 hr. 45 min.
2.....	195 c.c.	10 sec.	Neg.	Coag. in 21 min.	Coag. in 2 hrs. 54 min.
3.....	190 c.c.	15 sec.	Neg.	Coag. in 21 min.	Coag. in 1 hr. 55 min.
4.....	190 c.c.	14 sec.	Neg.	Coag. in 21 min.	Coag. in 3 hrs. 5 min.
5.....	150 c.c.	12 sec.	Neg.	Coag. in 21 min.	Coag. in 5 h. 53m.–6h. 8m.
6.....	130 c.c.	8 sec.	Neg.	Coag. in 22 min.	Neg. in 7 hrs. 15 min.

These results were rather surprising in that they seemed to indicate a clogging up of the pores of the filter by the rennet molecules and other substances, instead of the opening up of fresh channels. It will be observed, however, that in the former experiments which led to this series the intermediate filtrations were at 100 pounds' pressure, and not at 50 pounds.

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To determine the effect of the concentration of the solution upon the retention of the enzyme by the pores of the filter, the following experiment was performed. It tends to indicate that concentration is without effect in retarding the passage. If anything, it shows that the more concentrated the solution the more readily will the enzyme pass through.

D. Made up 250 c.c. of a 1:1,000 rennet solution and diluted 10 c.c. of this to 200 c.c. with 0.7 per cent. NaCl. Filtered 195 c.c. of the resulting 1:20,000 solution through the bougie used in *C*, at 50 pounds' pressure. Sterilized the bougie and 195 c.c. of the 1:1,000 solution were filtered through it at 50 pounds' pressure. Tests of filtrates and controls were made as before. The digestion temperature was 35°.

TABLE XXVI.

DILUTION	TIME OF FILTRATION	COAGULATING ACTION OF		
		Control		Filtrate
		Milk	Rennet	
1:20,000.....	11 sec.	Negative	Coag. in 34 min.	Coag. in 5½–7½ hrs.
1:1,000.....	11 sec.	Negative	Coag. in 2 min.	Coag. in 13 min.

Thus the passage through the bougie of rennet solutions at 50 pounds' pressure depends entirely upon the bougie employed. The enzyme is totally retained by a new bougie. When old bougies are used, the enzyme passes through in varying amounts, depending upon the previous use to which the filter has been put, and also upon the concentration of the enzyme solution.

EXPERIMENT VI.

Filtration at 100 pounds' pressure.—a) One hundred and ninety-five cubic centimeters of a 1:10,000 rennet solution were filtered through the Berkefeld bougie used in Experiment *A* of the 50-pound pressure series. The liquid passed through in seven seconds.

1 c.c. of control + 5 c.c. milk at 39°—coagulated in 16 min.
1 c.c. of filtrate + 5 c.c. milk at 39°—coagulated in 1½–1¾ hrs.
No milk control was made.

This showed that there was a partial retention of the enzyme by the filter. The bougie was washed by drawing through it about two liters of distilled water. It was then immersed in 50 c.c. of milk in a large test-tube, which was placed in an incubator at 37°. Beside it was placed another test-tube containing 50 c.c. of milk to serve as a control.

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The milk in the tube containing the bougie curdled at the end of six and one-half hours, whereas the control milk was still perfectly liquid. During the succeeding nine hours coagulation occurred also in the control tube, but at the end of that time the milk in the bougie tube showed a much more marked coagulation. The curd was solid and a clear transparent whey was present, whereas that of the control was creamy.

This experiment goes to show that the lessened activity of the filtrate is not due to the destruction of the enzyme, but that this is probably retained in an active condition in the pores of the filter.

b) In this test a new bougie was employed. The filtration of 195 c.c. at 100 pounds' pressure was not complete because the stopper blew out of the globe, but not, however, before 70 c.c. of the liquid had passed through. This passage was practically instantaneous.

1 c.c. control + 5 c.c. milk at 36° — coagulated in 17 min.
1 c.c. filtrate + 5 c.c. milk at 36° — negative in 7½ hrs.
No milk control was made.

This test shows a complete retention of the enzyme by the filter.

c) Through this same bougie the following series of filtrations at 100 pounds' pressure was carried on. After each filtration the bougie was washed and then immersed in 50 c.c. of milk. Duclaux[22] states that the Chamberland bougie immersed in milk will cause coagulation by surface action, layers of coagulum due to the rupture of the equilibrium of the milk particles by surface action being deposited on the bougie. In the following experiments, as in *a*, the action of the bougie was controlled by a sterilized Berkefeld bougie, similarly immersed.

TABLE XXVII.

(Digestion temperature, 36°.)

No.	Amt. of Filtrate	Time of Filtration	Coagulating Action of			Coagulating Action of Bougie	
			Control		Filtrate		
			Milk	Rennet		Control	Filter
1.....	190 c.c.	5 sec.	Negative	Coagulated in 14 min.	Coagulated in 5 hrs.	Negative	Coagulated in 5 hrs. 57 min.
2.....	194 c.c.	5-10 sec.	Negative	Coagulated in 21 min.	Coagulated in 1¼ hrs.	Negative	Coagulated in 8 hrs. 30 min.
3	195 c.c.	5-10 sec.	Negative	Coagulated in 26 min.	Coagulated in 43 min.	Negative	Coagulated in 8 hrs. 30 min.

It is thus seen that rennet is retained by a new Berkefeld filter even when filtered under a pressure of 100 pounds, but successive filtrations through the same bougie render it more permeable.

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EXPERIMENT VII.

A new Berkefeld bougie was rendered alkali-free, as before indicated. It was then used for a series of nine filtrations of a 1 : 10,000 rennet solution. These were made in three sets; the first set consisted of three filtrations at vacuum pressure, the second of a like number at 50 pounds' pressure, and the third at 100 pounds' pressure. For the first two sets two liters of a 1 : 10,000 rennet solution were made up. For the third set, performed on the next day, a liter of a fresh 1 : 10,000 solution was prepared. In each instance 195 c.c. were filtered, and 5 c.c. were set aside for control. The controls were subjected to the same light and temperature conditions as the filtering liquid. The control and the corresponding filtrate were tested at the same time. The tests were made as before at 35°, by adding 1 c.c. of the liquid to 5 c.c. of milk. Blank controls of the milk were made as before. *In no instance did the enzyme traverse the filter.*

TABLE XXVIII.

No.	Pressure	Time of Filtration	Coagulating Action of		
			Control		Filtrate
			Milk	Rennet	
1.....	27 in.	75 sec.	Negative	Coagulated in 18 min.	Negative in 13 hrs.
2.....	27 in.	75 sec,	Negative	Coagulated in 18 min.	Negative in 12 hrs.
3.....	27 in.	90 sec.	Negative	Coagulated in 18 min.	Negative in 9¼ hrs.
1.....	50 lbs.	16 sec.	Negative	Coagulated in 18 min.	Negative in 7¾ hrs.
2.....	50 lbs.	16 sec.	Negative	Coagulated in 17 min.	Negative in 6¼ hrs.
3.....	50 lbs.	17 sec.	Negative	Coagulated in 17 min.	Negative in 5¼ hrs.
1.....	100 lbs.	10–15 sec.	Negative	Coagulated in 17 min.	Negative in 11 hrs.
2.....	100 lbs.	*ca.* 10 sec.	Negative	Coagulated in 17 min.	Negative in 11 hrs.
3.....	100 lbs.	*ca.* 10 sec.	Negative	Coagulated in 17 min.	Negative in 9½ hrs.

When the 50-pound set was examined ten hours after the last observation indicated in the table, coagulation had occurred in both filtrate and in blank milk control tubes. The coagulum in the filtrate tubes did not resemble the rennet control coagulum, which formed, on standing a few hours, a thick curd, but resembled rather the creamy coagulum of the milk control.

At the end of twelve hours coagulation occurred simultaneously in both milk control and the filtrate tube of Filtrates 1 and 2 of the 100-pound set.

In every instance there is a total retention of rennet by the Berkefeld bougie at vacuum pressure, and, save in the case of old bougies, there is the same complete absence of activity in the filtrates of the 50-pound and 100-pound pressure filtrations.

The enzyme is not destroyed or altered by the bougie, but is merely retained mechanically by the pores of the filter. It is almost inconceivable that the enzyme, in an experiment such as *b* of the 100-pound filtrations, should become destroyed or chemi-

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cally altered during the almost instantaneous passage of the liquid through the bougie.

That the enzyme is not destroyed is evident from the fact that the bougies used in the 100-pound pressure filtrations, when immersed in milk, readily cause coagulation. That the pressure itself is without effect on the enzyme is clear from the demonstration that the same retention is observed in gravity filtration as in that at 100-pound pressure.

This fact is further shown by the following: 10 c.c. of a 1 : 10,000 solution of rennet were placed in a Pfungst autoclave and subjected to a pressure of 100 pounds for one hour at room temperature. In the meantime, as a control, 10 c.c. of the same solution were kept in a black box. At the end of the hour the coagulating action of each liquid was tested at 36°, with the following results:

1 c.c. autoclave rennet + 5 c.c. milk — coagulated in 34 min.
1 c.c. control rennet + 5 c.c. milk — coagulated in 34 min.
A milk control was negative.

It follows therefore that pressure is without effect.

EXPERIMENT VIII.

The following experiment was made in order to determine the effect, if any, exerted by the material composing the bougie upon the dissolved rennet:

A Berkefeld bougie was ground up in mortar to a very fine powder, which was then sterilized. To the powdered bougie in a beaker 190 c.c. of a 1:10,000 rennet solution were added, and the mixture was agitated by means of a mechanical stirrer for five minutes. A portion of 10 c.c. of the 1 : 10,000 solution was set aside as a control under the same temperature and light conditions as obtained for the mixture.

After the stirring ceased, the suspended particles settled very slowly and evenly. At the end of five and one-half hours the mixture was still very turbid. Ten cubic centimeters of mixture were then centrifugated, as also was the control, for thirty minutes. With the still cloudy liquid the following tests were made at 36°:

1 c.c. mixture + 5 c.c. milk—coagulated in 61 min.
1 c.c. control + 5 c.c. milk—coagulated in 36 min.
The milk control was negative.

After again sterilizing the powder, this experiment was repeated. The mixture was stirred this time for ten minutes and allowed to settle for two and three-quarter hours, after which it was centrifugated for one hour. A control solution was kept under the same conditions. The mixture remained turbid as in the preceding trial. The tests were made at 36°:

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1 c.c. mixture + 5 c.c. milk—coagulated in 57 min.
1 c.c. control + 5 c.c. milk—coagulated in 30 min.
The milk control was negative.

The decrease noted above in the activity of the rennet solution is no doubt due to the carrying down of the enzyme molecules by the sedimenting of the diatomaceous earth particles which make up the Berkefeld bougie.

EXPERIMENT IX.

It may be assumed that the behavior of the filter is due to the presence of some substance in the bougie which, when dissolved out by the rennet solution, inhibits the action of the enzyme.

If there is anything of an inhibiting nature in the undisintegrated bougie, this substance must clearly appear in the filtrate. The following tests show the filtrate to be perfectly free from any substance of an inhibitory nature:

One hundred and ninety-five cubic centimeters of a 1 : 10,000 rennet solution were filtered through a Berkefeld bougie at a pressure of 27⅞ inches in eighty-five seconds. Fifty cubic centimeters of filtrate were then added to 50 c.c. of unfiltered solution, and the mixture was subjected to mechanical stirring for ten minutes. Another portion of 50 c.c. of the unfiltered solution was set aside for control. The tests were then made at 35°:

1 c.c. mixture + 5 c.c. milk—coagulated in 46 min.
½ c.c. control + ½ c.c. distilled water + 5 c.c. milk—coagulated in 46 min.
1 c.c. control + 5 c.c. milk—coagulated in 23 min.
1 c.c. filtrate + 5 c.c. milk—negative in 7 hours.
The milk control was negative in 7 hours.

Repeated experiments produced the same results and confirmed the belief that the filtered liquid had no inhibitory action other than that of a mere diluent.

This fact was also shown in another way. One hundred cubic centimeters of the filtrate obtained in the above experiment were evaporated in a Roux vacuum desiccator at 37° to 10 c.c. The following mixtures were then made with a 1 : 10,000 rennet solution:

A = 5 c.c. evaporated filtrate + 5 c.c. rennet sol.
B = 5 c.c. distilled water + 5 c.c. rennet sol.
C = 1 c.c. evaporated filtrate + 5 c.c. rennet sol.
D = 1 c.c. distilled water + 5 c.c. rennet sol.

One cubic centimeter of each solution was then added to portions of 5 c.c. of milk, kept at 35°, with the following results:

1 c.c. A + 5 c.c. milk—coagulated in 58 min.
1 c.c. B + 5 c.c. milk—coagulated in 44 min.
1 c.c. C + 5 c.c. milk—coagulated in 34 min.
1 c.c. D + 5 c.c. milk—coagulated in 30 min.
The milk control was negative.

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The controls *B* and *D* are not strictly comparable to the test mixtures since they were made with distilled water, whereas the evaporated filtrate, owing to the fact that the rennet solutions were made up in physiological salt, contained seven per cent. of NaCl. To show the effect of seven per cent. of NaCl on a 1 : 10,000 rennet solution, the following mixtures were made and tested at 35°, as before:

A = 5 c.c. 7 per cent. NaCl sol. + 5 c.c. rennet sol.
B = 5 c.c. distilled water + 5 c.c. rennet sol.
C = 1 c.c. 7 per cent. NaCl sol. + 5 c.c. rennet sol.
D = 1 c.c. distilled water + 5 c.c. rennet sol.

Tested as follows:

	First Test	Second Test
1 c.c. *A* + 5 c.c. milk	coagulated in 66 min.	coagulated in 67 min.
1 c.c. *B* + 5 c.c. milk	coagulated in 50 min.	coagulated in 51 min.
1 c.c. *C* + 5 c.c. milk	coagulated in 30 min.	coagulated in 32 min.
1 c.c. *D* + 5 c.c. milk	coagulated in 30 min.	coagulated in 30 min.

It is thus seen that the retarding action of the evaporated filtrate in this experiment is due to the presence of seven per cent. NaCl.*

EXPERIMENT X.

Filtration through Chamberland filter.—The series of filtrations tabulated below show that the rennet molecule is also unable to pass through the pores of the Pasteur filter, size *F*. The bougie before use was thoroughly cleansed, ignited, and sterilized by autoclaving before each individual filtration. For each filtration at vacuum pressure 190 c.c. of a 1 : 10,000 rennet solution were employed. The filtrations at 50 pounds' pressure were made with 195 c.c. The filtrates were all tested for alkalinity and found to be neutral in reaction. The controls and tests, at 36°, were made as in the case of the Berkefeld filtrations.

TABLE XXIX.

No.	Pressure	Time of Filtration	Coagulating Action of		
			Control		Filtrate
			Milk	Rennet	
1.....	27½-27⅞ in.	3¼ min.	Negative	Coag. in 25 min	Neg. in 10¼ hrs.
2.....	27⅞ in.	3⅓ min.	Negative	Coag. in 22 min.	Neg. in 9¼ hrs.
3.....	27½-28⅛ in.	4 min.	Negative	Coag. in 23 min.	Neg. in 7 hrs.
1.....	50 lbs.	80 sec.	Negative	Coag. in 20 min.	Neg. in 13 hrs, 10 min.
2.....	50 lbs.	70 sec.	Negative	Coag. in 20 min.	Neg. in 11 hrs. 30 min.
3.....	50 lbs.	75 sec.	Negative	Coag. in 14 min.	Neg. in 10 hrs.

* *Cf.* OPPENHEIMER, *Die Fermente, etc.*, p. 149.

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EXPERIMENT XI.

Filtration through collodion sacs.—Filtration through collodion sacs (as previously described) resulted in the absolute retention of the enzyme by the filter.

The filtrates and controls were tested at 36° as in previous experiments.

TABLE XXX.

No.	Dilution	Pressure	Time of Filtration	Am't Filtered	Coagulating Action of		
					Control		Filtrate
					Milk	Rennet	
1.....	1:10,000	2-3 in.	2 hrs.	6½ c.c.	Negative	Coag. in 23 min.	Neg. in 6 hrs.
2.....	1:1,000	2½ in.	2¼ hrs.	4 c.c.	Negative	Coag. in 2 min.	Neg. in 8½ hrs.
3.....	1:1,000	4 in.	1 hr.	4 c.c.	Negative	Coag. in 3 min.	Neg. in 9 hrs.

EXPERIMENT XII.

Dialysis through collodion sacs.— It will be seen from the following, which is one of several similar experiments, that rennet dialyzes readily through the collodion sac.

Seven very thin sacs were prepared by rolling the tube used in their preparation twice in thin collodion. These were designated *C*, 1, 2, 3, 4, 5, and 6. Five cubic centimeters of a 1:1,000 rennet solution, previously boiled for five minutes, were placed in the sac marked *C*, and served as a control. In each of sacs 1–6, inclusive, were placed 5 c.c. of a fresh 1:1,000 solution. Each sac was then immersed in a test-tube on foot containing 25 c.c. of milk and placed in a nincubator at 36°. The first evidence that the enzyme dialyzed through the walls of the sac was seen in the formation of a coagulum which adhered in clumps to the outside of the sac. This "bushing" increased in extent until in time the entire contents of the tube coagulated. The results obtained were as follows:

Slight "bushing" in Nos. 1, 3, 5, and 6 in 6 hrs.
Marked "bushing" in Nos. 1, 2, 3, 4, and 5 in 9 hrs.
Slight "bushing" in No. 6 in 9 hrs.
No "bushing" in control in 9 hrs.

When next examined at the end of twenty-one hours, the control showed a soft, creamy coagulum extending through the mass of milk, whereas in Tubes 1–6, inclusive, the curd formed a solid layer at the bottom of the tube, while above it was a layer of clear liquid. Solid masses of curd were adherent to each sac.

The sacs employed in this experiment were tested before and after use, and were found to be free from leaks.

As in the case of ptyalin, dialysis was shown to occur through the same sacs, which did not allow filtration of the enzyme.

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By way of contrast to the behavior of the collodion sac may be given an experiment wherein dialysis was attempted by placing within a Berkefeld bougie 10 c.c. of a 1:10,000 rennet. The bougie was placed in a test-tube in 40 c.c. of milk and set aside at 36°. No dialysis occurred; the result in this case agreeing with that of Lea and Dickinson (*vide supra*).

The foregoing study of the behavior of rennet shows (1) that it is removed from its solution by filtration through hardened filter paper; (2) that this removal is not due to fixation by the paper; (3) that it is readily destroyed by aeration and surface action; (4) that it is removed completely from its solution by the pores of a new Berkefeld bougie, even under high pressure; (5) that this removal is mechanical and is not due to chemical destruction; (6) that it is removed in like manner by the Chamberland filter; (7) that it readily dialyzes through the collodion sac, but not through a Berkefeld bougie; (8) that it is removed from its solution when filtered through a collodion sac.

PEPSIN.

PAPER FILTRATION.

The pepsin used in these experiments was that of Parke, Davis & Co. The solutions were prepared by dissolving the preparation in 0.7 per cent. NaCl. They were filtered before use, either before or after dilution, through a single thickness of ordinary filter paper. The action of filtrates, controls, etc., in these experiments was tested by adding 0.1 g. of fibrin to 5 c.c. of the pepsin solution, to which had been added 1 c.c. of a 10 per cent. HCl. This fibrin was obtained from hog's blood and was well washed first in running water, then in distilled water, and was carefully pressed, just before weighing, between several thicknesses of filter paper, in order to remove as much moisture as possible.

EXPERIMENT I.

The following table gives the results of three sets of filtrations of a 1:16,000 pepsin solution. A common control was used for the "6×2" and "8×2" filtrates. The arrangement of filters, it may be added, was the same as that heretofore employed.

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TABLE XXXI.

No.	Duration of Filtration	Digestion Temperature	Time Required to Produce Solution of Fibrin by						
			"2×2"		"4×2"		"6×2"		"8×2"
			Control	Filtrate	Control	Filtrate	Control	Filtrate	Filtrate
1...	2 hrs.	37°	5 hrs.	5 hrs.+	3-4 hrs.	4-5 hrs.	3 hrs.	Neg. in 24 hrs.	Neg. in 24 hrs.
2...	2 hrs. 35 min.	38°	2-3 hrs.	3 hrs.+	2-3 hrs.	¾ dissol. in 18 hrs.	2-3 hrs.	Neg. in 18 hrs.	Neg. in 18 hrs.
3...	1 hr. 50 min.	35°	¾ dissol. in 4 hrs.	4 hrs.	¾ dissol. in 4 hrs.	6-7 hrs.	¾ dissol. in 3 hrs.	¾ dissol. in 11 hrs.	¾ dissol. in 11 hrs.

A blank control was made in each case with 0.1 g. fibrin in 5 c.c. of 0.7 per cent. NaCl containing 0.1 c.c. of a 10 per cent. HCl. These were uniformly negative during the time of the experiment.

This experiment clearly shows that pepsin is removed from its solution by hardened filter paper.

To determine if this decrease in the activity of the enzyme solutions was due to fixation by the filter paper, experiments were performed similar to those demonstrating the fixation of ptyalin, but no fixation could be demonstrated. Aeration was observed to have a destructive effect upon the enzyme, and this effect was aided by the surface action of the paper bits or garnets over which the pepsin was aerated.

EXPERIMENT II.

The effect of Berkefeld filters.— The table below shows the effect of Berkefeld filtration on 1:16,000 pepsin solutions. One hundred and ninety cubic centimeters were filtered in each instance. The filtrate and control tests and blank controls were made as before. The digestion was carried on at 36–37°. The bougie was sterilized before each filtration by autoclaving.

TABLE XXXII.

No.	Pressure	Time of Filtration	Time Required to Produce Solution of Fibrin by		
			Control		Filtrate
			Blank	Pepsin	
1.....	27⅞ in.	65 sec.	Negative	1-2 hrs.	10-20 hrs.
2.....	27¾ in.	65 sec.	Negative	*ca.* 1½ hrs.	8-18 hrs.
3.....	27¾ in.	65 sec.	Negative	*ca.* 1½ hrs.	½ dissol. in 10 hrs.
4.....	50 lbs.	16 sec.	Negative	1½-2 hrs.	12 hrs. +
5.....	50 lbs.	16 sec.	Negative	*ca.* 2 hrs.	¾ dissol. in 18 hrs.
6.....	50 lbs.	20 sec.	Negative	*ca.* 2 hrs.	¾ dissol. in 18 hrs.
7.....	100 lbs.	5 sec.	Negative	*ca.* 2 hrs.	¾ dissol. in 24 hrs. +
8.....	100 lbs.	10 sec.	Negative	*ca.* 2 hrs.	¾ dissol. in 24 hrs.
9.....	100 lbs.	8 sec.	Negative	2-3 hrs.	¾ dissol. in 24 hrs.

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In Tests 1–4, inclusive, hog fibrin several days old was used.

The two following filtrations were made with the same bougie used above. The tests were carried out at 36°.

TABLE XXXIII.

No.	Pressure	Time of Filtration	Solvent Action on Fibrin of		
			Control		Filtrate
			Blank	Pepsin	
1.....	28 in.	70 sec.	Neg.	Solution in 2–3 hrs.	Negative in 15 hrs.
2.....	27½ in.	70 sec.	Neg.	Solution in 2–3 hrs.	Negative in 14 hrs.

In Table XXXII a less ready passage is observed at 100 pounds' pressure than at vacuum pressure. The subsequent filtrations through the same bougie at vacuum pressure indicated a clogging of the pores of the filter by pepsin and other substances in the successive filtrations. Fermi and Pernossi (*loc. cit.*) call attention to the fact that the passage of pepsin through the Chamberland bougie is less ready when the bougie used had previously been employed for the filtration of colloidal substances.

These experiments go to show that a marked difference exists between rennet and pepsin in regard to the passage through the Berkefeld filter.

EXPERIMENT III.

Filtration through collodion sac.—In a series of trials it was shown that pepsin does not pass through the collodion filter. The results are given in the following table. The pepsin solutions employed were 1:16,000 and the digestion was carried on at 37°.

TABLE XXXIV.

No.	Pressure	Am't Filtered	Time of Filtration	Time Required to Dissolve Fibrin by		
				Control		Filtrate
				Blank	Pepsin	
1.....	2½–3 in.	5 c.c.	45 min.	Partial 24–38 hrs.	*ca.* 2 hrs.	Neg. in 38 hrs.
2.....	1–3 in.	....	1¼ hrs.	Neg. in 34 hrs.	2 hrs.	Neg. in 34 hrs.
3.....	2¼ in. (*ca.*)	8 c.c.	30 min.	Neg. in 48 hrs.	2–3 hrs.	Sol. in 24 hrs.
4.....	2 in. (*ca.*)	6 c.c.	19 min.	Neg. in 2¼ hrs.	*ca.* 2 hrs.	Sol. in 2¼ hrs.
5.....	1–2¼ in.	4 c.c.	2¼ hrs.	Neg. in 24 hrs.	*ca.* 2 hrs.	Neg. in 24 hrs.
6.....	1–3 in.	4 c.c.	2¼ hrs.	Neg. in 24 hrs.	*ca.* 2 hrs.	Neg. in 24 hrs.

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The rapidity of the filtration process in trials Nos. 3 and 4 made it quite probable that some leakage of the sacs occurred. With the exception of these two, the results indicate that pepsin cannot pass through the collodion filter.

EXPERIMENT IV.

Dialysis through collodion sacs.—As with other enzymes heretofore stated it will be seen that although pepsin does not filter through the collodion wall, it is dialyzable through that membrane. The following experiment will illustrate the dialysis of pepsin:

Seven thin collodion sacs were prepared as before described and were labeled *S*, *C*, *C'*, 1, 2, 3, 4, and 5, respectively. Each sac was immersed in a test-tube on foot containing 20 c.c. of a 0.7 per cent. NaCl, acidulated with 0.4 c.c. of a 10 per cent. HCl, and 0.1 g. fibrin. Each of the sacs *C* and *C'* contained 5 c.c. of a 1:5,000 pepsin solution, previously boiled for five minutes, and served as controls. In each of sacs 1–5, inclusive, were placed 5 c.c. of a freshly prepared 1:5,000 pepsin solution. All the tubes were set aside in an incubator at 37°.

When observed at the end of twenty-eight hours, the solution of the fibrin was complete in Tube 4. A small sediment remained in Nos. 1, 2, and 5. A small lump of undissolved fibrin remained in No. 3. The fibrin placed in this tube consisted of a solid piece, and not of shreds as in the other tubes. The controls showed no solution. At end of forty-eight hours the solution of the fibrin in sacs 1–5, inclusive, was practically complete, whereas in the control tubes there remained a comparatively large sediment.

The sacs were tested at the close of the experiment, and were found free from leaks.

The foregoing details show (1) that pepsin is removed from dilute solution by filtration through hardened filtered paper; (2) that this removal is probably not due to fixation by the paper; (3) that aeration and surface action are injurious to pepsin; (4) that pepsin is removed from solution by the Berkefeld bougie and by the collodion filter; (5) and that it readily dialyzes through the collodion sac.

PANCREATIN.

The pancreatin used in these experiments was the commercial preparation of Parke, Davis & Co. The solutions employed, except when otherwise indicated, were of a strength of 1 : 500 in 0.7 per cent. NaCl. Before use they were filtered through a single thickness of ordinary filter paper.

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The pancreatin was tested by adding to 5 c.c. of the solution 0.08 c.c. of a 10 per cent. Na_2CO_3, and 0.1 g. fibrin. One hundred and ninety cubic centimeters were filtered in each instance. Control tests were made with the unfiltered solution. Blank control consisted of 0.1 g. fibrin in 5 c.c. of a 0.7 per cent. NaCl containing 0.08 c.c. of a 10 per cent. Na_2CO_3.

EXPERIMENT I.

Filtration through paper.—The following table gives the results obtained on filtering the pancreatin solution through a series of hardened filter paper. The tests were made at a temperature of 36°. A common control served for the "6 × 2" and "8 × 2" filtrates. Blank controls, it may be added, were negative in every trial.

TABLE XXXV.

No.	Duration of Filtration	Time Required to Dissolve Fibrin by						
		"2 × 2"		"4 × 2"		"6 × 2"		"8 × 2"
		Control	Filtrate	Control	Filtrate	Control	Filtrate	Filtrate
1.....	3 hrs. 15 min.	8–9 hrs.	*ca.* 10 hrs	8–9 hrs.	10–18 hrs.	8–9 hrs.	31–42 hrs.	Neg., 18 hrs
2.....	2 hrs. 15 min.	7–10 hrs.	7–10 hrs.	*ca.* 10 hrs	10–21 hrs.	9 hrs.	22–23 hrs.	Neg., 48 hrs
3.....	2 hrs. 20 min.	7¼–9 hrs.	*ca.* 9 hrs.	7–8 hrs.	8–21 hrs.	6–7¾ hrs.	21–24 hrs.	Sol. in 48–72 hrs.

It is evident from these tests that pancreatin is removed from its solution by hardened filter paper.

Subsequent aeration experiments failed to demonstrate the fixation of enzyme. Aeration was found to have a destructive effect on the enzyme, which destructive effect was increased by the surface action of paper and of garnets.

EXPERIMENT II.

Filtration through the Berkefeld filter.—The results obtained by Fermi and Pernossi and others, in connection with the filtration of pancreatin through porous filters have already been noted. Below are tabulated the results of a series of filtrations through the Berkefeld bougie. The filtrates and controls were tested in the manner described above. One hundred and ninety cubic centimeters of the 1 : 500 enzyme solution were filtered in each instance. The digestion temperature was 36–37°.

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TABLE XXXVI.

No.	Pressure	Time of Filtration	Time Required to Produce Solution of Fibrin by		
			Control		Filtrate
			Blank	Pancreatin	
1.....	22¾ in.	40 sec.	Negative	5 hrs.	24–25 hrs.
2.....	28⅛ in.	55 sec.	Negative	7+ hrs.	24–30 hrs.
3.....	28 in.	75 sec.	Negative	8 hrs.	¾ diss. in 48 hrs.
4.....	50 lbs.	16 sec.	Negative	9½–11½ hrs.	24 hrs.
5.....	50 lbs.	16 sec.	Negative	8¼–10¼ hrs.	35 hrs.
6.....	100 lbs.	About 12 sec.	Negative	8 hrs.	50 hrs.
7.....	100 lbs.	About 10 sec.	Negative	8 hrs.	30–45 hrs.
8.....	100 lbs.	About 10 sec.	Negative	8 hrs.	30–45 hrs.

It will be observed that, as in the case of pepsin, the bougie becomes clogged by successive filtration.

After the second filtration at 50 pounds' pressure, the bougie in use was accidentally broken. The subsequent filtrations at 100 pounds were then performed with a new bougie. During the process of filtration the tube connecting the filtering apparatus with the pressure tank broke, after 98 c.c. of the liquid had passed through. The succeeding portion of liquid which filtered through at gravity pressure was collected separately. The time of filtration at 50 pounds' pressure was seven or eight seconds. The gravity filtration which followed gave 3–4 c.c. per minute. The filtrates and controls were tested as before with the following results:

Blank control — negative in 48 hrs.
Pancreatin control — solution in 10–21 hrs.
Gravity filtrate — negative in 48 hours.
50 pounds' filtrate — negative in 48 hrs.

Evidences of bacterial growth in these tubes appeared at the end of forty-eight hours, and hence the observations were discontinued. There was in this instance no passage of pancreatin through the bougie. This same bougie was used in 100-pound filtrations tabulated above.

EXPERIMENT III.

Filtration through collodion sacs.—The collodion filter is impermeable to trypsin as the following table will show.

TABLE XXXVII.

No.	Pressure	Time of Filtration	Amount Filtered	Temp. of Digestion	Time Required to Dissolve Fibrin by		
					Control		Filtrate
					Blank	Pancreatin	
1.....	1½–3 in.	2 hrs.	5 c.c.	36°	Neg.	6½–8 hrs.	Neg. in 48 hrs.
2.....	2–3⅛ in.	2 hrs.	5¼ c.c.	37°	Neg.	7+ hrs.	Neg. in 72 hrs.
3.....	2–2⅛ in.	1½ hrs.	5⅛ c.c.	37–38°	Neg.	*ca.* 8 hrs.	Neg. in 72 hrs.

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Dialysis through collodion sacs.—Unlike the enzymes already studied, pancreatin does not dialyze through the collodion sac. The dialysis experiments were performed exactly as in the case of preceding enzymes. The tubes were kept under observation in one instance as long as eleven days, but at no time was there any evidence of dialysis.

Inasmuch as the commercial pancreatin was employed for the preceding experiments, it was deemed desirable to check these with a freshly prepared trypsin solution. Accordingly, a fresh beef pancreas was ground up in a sausage machine, and the pulp was extracted with water. The filtered solution was precipitated with alcohol, and the precipitate was then digested with glycerin. The glycerin extract thus obtained was found to possess a marked proteolytic action. All attempts to dialyze this trypsin solution or to filter through the collodion sac gave negative results, as did the commercial preparation.

These experiments show (1) that pancreatin is removed from its solution by filtration through hardened filter paper; (2) that this removal evidently is not due to fixation; (3) that the enzyme is injuriously affected by aeration and by surface action; (4) that it passes in varying degree through the Berkefeld bougie, in one instance being totally retained; (5) that it does not filter through the collodion sac; (6) and that, unlike all the other enzymes experimented upon, it does not dialyze through the collodion sac.

CONCLUSIONS.

The results obtained in this study may be advantageously and briefly summarized as follows:

I. The removal from solution by paper filtration of ptyalin, rennet, pepsin, and pancreatin.

II. The fixation of ptyalin by hardened filter paper.

III. The injurious effect of aeration and of surface action on taka diastase, rennet, pepsin, and pancreatin.

IV. The passage of ptyalin and taka diastase through the Berkefeld bougie; the absolute retention of rennet, even under high pressure, by the Berkefeld bougie; the partial retention of pepsin; and the partial retention (in one case total retention) of pancreatin.

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V. The total retention of rennet by the Chamberland bougie.

VI. The total retention of ptyalin, rennet, pepsin, and pancreatin by the collodion filter, and the partial passage through this filter of taka diastase.

VII. The dialysis of ptyalin, taka diastase, rennet, and pepsin, but not of pancreatin, through the collodion membrane.

I wish to express my obligation to Professor Novy, at whose instance this work was undertaken, and whose advice and suggestions have been of valuable assistance throughout the work. Acknowledgments are also due to the Rockefeller Institute for Medical Research for the support necessary to carry on this investigation.

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Reprinted from
THE JOURNAL OF INFECTIOUS DISEASES, Vol. II, No. 2, March 1, 1905, pp. 256–308

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ON THE TRYPANOSOMES OF BIRDS.*†

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Explanation of Plates.

INTRODUCTION.

THE problem of the cultivation of the malarial parasites, obviously one of great importance, is as yet untouched. The appearance of Schaudinn's remarkable paper[85]‡ in the early part of 1904 seemed to assure its definite solution. For, if the conclusions arrived at by this eminent investigator were found to be correct, all that would be necessary would be to cultivate the trypanosome stages by means of the method which has proved so successful with *Tr. Lewisi*, *Tr. Brucei*, and *Tr. Evansi*, and the problem would be solved.

It is but fair to say that we entered upon this work with the fullest confidence in the correctness of the conclusions reached by

* Received for publication January 8, 1905.

† This paper will be followed, in the next number of this *Journal*, by a second in which the other hematozoa of birds will be considered. A summary of the main results arrived at was presented before the International Congress of Arts and Science at St. Louis, September 23, and before the Society of Experimental Biology and Medicine, October 19, and was published in *American Medicine*, November 26.

‡ The references to the literature will be given in the paper "On the Hematozoa of Birds."

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Schaudinn. So much so was this the case that our early results were interpreted at first as fully upholding his views. As the work progressed, however, it was found that the results obtained, instead of supporting, actually contradicted his conclusions.

Schaudinn's extensive memoir must be read in the original by those who are interested in the subject. The following summary will, however, serve to bring out the main points in his paper.

On allowing the common mosquito (*Culex pipiens*) to feed upon the blood of owls infected with *halteridium* (*Hæmoproteus noctuæ*) he found in the intestines of about 10 per cent of these insects large numbers of trypanosomes which he considered as stages in the life-history of this hemocytozoön. The oökinetes, resulting from the fertilization of macrogametes, showed from the start evidence of sexual differentiation. Thus, one type changed to an indifferent or asexual trypanosome, which then rapidly multiplied by longitudinal division, that is, in the same manner as do ordinary trypanosomes. Another type of oökinete gave rise to a female trypanosome which apparently was incapable of multiplying by longitudinal division, but by parthenogenesis regenerated all three kinds of trypanosomes. The third type of oökinete gave rise directly to eight male trypanosomes or microgametes, which, as such, are incapable of further development or multiplication, and merely serve to fertilize the macrogametes. This type of oökinete therefore corresponds to the free microgametocyte of the blood. All three forms of trypanosomes under unfavorable conditions agglutinate, forming rosettes with centrally directed flagella.

The indifferent trypanosomes, as a result of rapid multiplication, become very small, and when injected into the blood of an owl attach themselves by the flagellar end to the erythrocytes. The flagellum is then absorbed and the parasite appears as the well-known early stage of the *halteridium*. Usually during the night, the parasite leaves the surface of the red blood cell, at first, as a gregarine-like vermiculus which later takes on the trypanosome form. This, after a period of motion, again attaches itself to a cell and passes into a condition of rest, during which it grows until the next night when it again leaves its host. This alternation of the motile and rest periods continues until the sixth day when the *halteridium* has reached its full size, whereupon it leaves the cell and by rapid, consecutive, longitudinal division gives rise to small flagellates which then begin a new six-day cycle. The indifferent trypanosome in this way gives rise to the male and female *halteridia* of the blood.

The female trypanosomes when introduced into the blood likewise attach themselves to erythrocytes, but change their host-cells less often than do the former. When they leave the cell they do not take on the trypanosome form, but remain as gregarine-like vermiculi, which after a while enter new cells. The fully developed form appears as the typical macrogamete which no longer is able to leave the cell.

The male trypanosome or microgamete, when introduced into the blood, dies out rapidly. The male and female types result from and are constantly renewed by the differentiation of the indifferent trypanosomes or *halteridia*.

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The results obtained by Schaudinn with the "leucocytozoön" of Danilewsky (*Hæmamœba Ziemanni*) were much the same as those mentioned above for the *halteridium*. When the blood of the owl which contains this parasite is sucked up by the mosquito (*Culex pipiens*) fertilization of the sexual elements occurs in the gut of the mosquito in the same way as under a cover-glass. The female cell or macrogamete ripens and becomes free; the microgametes are given off from the male cell or microgametocyte and fertilize the female cell. A large oökinete results which then grows in length — giving rise to a complicated coil. At the same time the original nucleus divides in rapid succession, and thus arise a large number of nuclei each of which becomes surrounded by a dense plasma zone. These small cell-territories, as in the case of the oökinete of the *halteridium*, develop into trypanosomes which become indifferent, male or female in type, according to the character of the original oökinete. These trypanosomes eventually swarm out of the large coils or differentiated oökinetes.

The indifferent forms have all the structure of typical trypanosomes. They multiply by longitudinal division and with such rapidity that the resulting young cells become extremely small; so small indeed as to be scarcely visible. Schaudinn expressed the belief that these small forms could readily pass through a Chamberland filter. When a cell divides, the two daughter cells remain attached by the posterior ends. Another feature to which Schaudinn calls special attention is agglutination. The resulting clumps are made up of indifferent cells which come together by their posterior ends, and hence their flagella are on the outside of the rosette, whereas in the case of the *halteridium* trypanosome the whips are directed centrally. It is because of this fact that Schaudinn designates this type as a "*spirochete*."

The female spirochetes are larger than the indifferent forms; the plasma is darker, the nucleus and blepharoplast are relatively small, and the undulating membrane is not prolonged as a free flagellum. The male spirochetes are so small as to be scarcely visible.

The sexual forms develop from the indifferent types. Owing to their rapid growth they soon become larger than their host-cells, and are therefore unable to enter these.

Such a spirochete, therefore, attaches itself by its posterior end to an erythrocyte which it then draws into itself. After it digests the plasma the nucleus is pushed off to one side as an elongated halter-shaped body, eventually to be thrown away as waste. This very novel view of the structure of the leucocytozoön is at variance with our own observations.

It is worthy of note that Schaudinn regards the spirochetes of recurrent fever and of geese as essentially the same as the preceding. There can be but little doubt that these organisms will be shown to be trypanosomes and hence unrelated to the bacteria.

It is evident from Schaudinn's work that he holds that such trypanosomes as chance to be present in the blood of birds represent not distinct species of organisms but merely stages in the development of intracellular parasites. This radical view, how-

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ever, cannot be substantiated, for it will be shown that trypanosomatic infection is very common among birds, and that it bears no relation to the intracellular parasites which may or may not be present. Moreover, his two types of trypanosome and spirochete are really two forms of one species.

The reason for this difference in the conclusions arrived at by Schaudinn and ourselves lies clearly in the methods employed. There can be no question as to the fact that Schaudinn cultivated trypanosomes, *in vivo*, in the mosquito, but since this insect can be infected by several malarial parasites it follows that he really worked with *mixed cultures*, if we may thus use this term. The apparently positive results obtained by the injections of suspensions of the infected mosquitoes must be considered as due, not to the trypanosomes which chanced to be present, but to the unrecognized developmental forms of the hemocytozoa.

On the other hand, we cultivated the trypanosomes *in vitro*, under which conditions the trypanosomes multiply with great readiness, whereas the hemocytozoa die out. We thus obtained from a series of birds a large number of *pure cultures* of trypanosomes, representing several species. With such material we were unable to infect birds so as to give rise to intracellular parasites. As in the case of bacteria, the pure culture is after all the means that enables one to solve the relation of a protozoön to a certain phenomenon, which, by assumption, it may be credited to possess.

OCCURRENCE OF TRYPANOSOMES IN BIRDS.

The earliest observation on the presence of protozoa in birds is perhaps that of Gros,[34] who described organisms in the blood of the crane, crow, and fern-owl. His flagellates were 10–15 μ long, and very narrow (microgametes ?). In the crow he found a hematozoön which was 100–130 μ long, and narrower than a red blood cell. It is more likely that this was a filaria rather than a trypanosome.

Similarly, Wedl's observations[93] leave it by no means certain that he saw trypanosomes. In the fresh blood of the cherry-finch (*Loxia coccothraustes*) he found filaria and oval bodies of about the size of red blood cells. These bodies were provided at one end with a crown of cilia, and were regarded by Danilewsky as trypanomonas.

In view of the indefiniteness of these early observations it is but proper to credit Danilewsky with the discovery of bird trypanosomes. His first paper[8] on this subject appeared in 1885, and four years later a very extended description was published in book form.[12]

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Danilewsky examined more than 300 birds, but gave no details as to the frequency and number of the trypanosomes found by him. They were met with in the blood of owls, rollers, lannerets, etc., and the number was said to vary with the individuals and the season. In one case they were found in young rollers (*Coracias garula*) but three or four days old. They were also found in young featherless lannerets. He observed only one form of trypanosome which to him corresponded perfectly with the *Tr. fusiforme* (*piscium*) of fish. According to the size he divided this into *Tr. majus* and *Tr. minus*. The length of the latter, not counting the flagellum, varied from 18–22 μ; while that of the former was 45–60 μ. The young forms (9–10 μ), arising by segmentation, he designated as *trypanomonas*. Whereas in the heart-blood but one or two trypanosomes could be found, and then only with difficulty, in the red marrow of bones they were detected in large numbers. It would seem as if the red marrow was the principal place where these organisms are found.

Although Danilewsky designated the trypanosomes by several names, such as *Tr. fusiforme*, *Tr. majus*, *Tr. minus*, *Tr. sanguinis avium*, *Tr. fusiforme avium*, *Tr. costatum*, it must not be assumed that these meant to him distinct species. On the contrary he held to a unity among the trypanosomes just as he did for the cytozoa of birds. Nevertheless, it is evident that he met with at least two distinct forms in respect to size. Our own results tend to confirm his view that these two forms represent stages in the development of one and the same species.

Grassi and Feletti[29] mention the presence of trypanosomes in bats, but accord no notice to those of birds, other than to express their conviction that they have nothing to do with the malarial parasites.

According to Sjöbring[88] trypanosome infection of birds is widespread about Säftstaholm in Södermannland, Sweden. He was able to find the organism in nearly all passerine birds examined by him with the exception of Corvus and Pica. The infection was apparently local, for elsewhere he did not meet with it. Beyond this mere statement he gave no detail, and merely added that the forms observed corresponded with those of Danilewsky.

Ziemann,[94] in 1898, in 190 birds examined found trypanosomes but once and that in a chaffinch (*Fringilla cœlebs*) from Heligoland. Beyond the mere mention of this fact nothing further is said. The title of a more recent paper by Ziemann,[95] which indicates the presence of a trypanosome in an owl captured in Cameroon, is misleading. As a matter of fact, he did not find a real trypanosome but, acting upon a personal communication from Schaudinn, he used this term to designate a "leucocytozoön."

According to Laveran,[55] trypanosomes are very rare in birds indigenous to France, inasmuch as he examined a large number with negative results. In an owl (*Syrnium aluco*), purchased in Paris, he found trypanosomes, but they were very scarce. This bird had a quadruple infection: filaria, *H. Danilewskyi*, *H. Ziemanni*, and Trypanosoma. The latter, whip included, measured 33–45 μ. Following Danilewsky, Laveran designated this species as *Tr. avium* and carefully described its characteristics.

Dutton and Todd,[25] on their expedition to Senegambia, were able to find a larger number of trypanosomatic infections than any of their predecessors. Of 40 birds examined eight were found to have trypanosomes in their blood.

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At Bathurst, 25 birds (mostly *Estrelda* and *Crithagra*) were examined and of these only one was infected. The scarcity of the parasites in the blood may be seen from the fact that they found but from two to four trypanosomes in a cover-glass preparation. This particular species they designated as *Tr. Johnstoni*. It had a very active spirillum-like motion, but possessed no free flagellum, and the undulating membrane was scarcely recognizable. The body was long, straight, and pointed at both ends. It measured 36–38 μ in length, and 1.4–1.6 μ in width. Two larks were inoculated with the blood from this bird, but with negative result.

At St. Louis, Senegal, they examined 15 birds (*Estrelda* and *Crithagra*) and found seven of these to be infected. The species in this case was left undetermined. They were not present in large numbers, were stumpy, sluggish, and of great width. The body measured 21.6 by 8 μ; the length of the flagellum was 10–12 μ. Two pigeons and two larks were inoculated with negative results.

Hanna,[85] while in India in 1900, examined the blood of domestic pigeons which were infected with *H. Danilewskyi*, and found trypanosomes. The percentage of infected birds was not large, and the parasites were comparatively few in number. The health of the birds was not affected. The trypanosome described and figured by Hanna is 45–60 μ in length, and 6–8 μ in width. A feature which distinguishes this trypanosome from that of Laveran is the transverse position of the nucleus and the nearness to this of the blepharoplast. In this respect it agrees, it may be added parenthetically, with one of the forms obtained by ourselves.

In this same paper Hanna gives a description of some trypanosomes present in preparations made from the blood of crows by Ross, while in India, in 1898. Apparently the two forms represent different species as can be seen from the following measurements:

	Pigeon	Crow
Length of organism	45–60 μ	40–56 μ
Breadth opposite to nucleus	6–8 μ	3–4.8 μ
Length from centrosome to posterior end	19–22 μ	8–9.5 μ

In this connection it may be stated that Laveran and Mesnil[57] cite a personal communication from Donovan, who found trypanosomes at Madras in the blood of an owl (*Athene brama*).

In 1903 Edm. and Et. Sergent[86] examined 307 birds in Algeria. It is worthy of note that all but 18 harbored one or more parasites. Thus, 37 had *Hæmamœba relicta*, 155 had *H. Danilewskyi*, two had *H. Ziemanni*, 42 had filaria, and six had trypanosomes. The latter organisms were extremely rare, and were found only in the fresh blood, not in the stained preparations. We have found this to be usually the case in our own examinations. The trypanosomes were present in one out of 46 goldfinches (*Fringilla carduelis*), in two out of five black-caps (*Sylvia atricapilla*), and in three out of 10 swallows (*Hirundo*) examined. No detailed descriptions or measurements are given.

Early in 1904 Schaudinn[85] reported his studies upon the owl (*Athene noctua*), in which he found proteosoma, halteridium, and *H. Ziemanni*. The last two organisms, for reasons already given, he designated as *Trypanosoma*

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noctuæ and *Spirochæta Ziemanni* respectively. By inference, rather than from actual statement, it is clear that trypanosomes were present in the naturally infected birds.

This belief is substantiated by the recent work (July, 1904) of the Sergents[87] upon owls in Algeria. They were able this time to find trypanosomes, in very small numbers, in stained preparations, and confirmed Schaudinn's observations with reference to the presence of trypanosomes in the digestive canal of mosquitoes which sucked the blood of this owl.

The presence of trypanosomes in the stomach of mosquitoes was observed prior to the work of Schaudinn and of the Sergents. Thus, Chatterjee[6] is quoted by Rogers[76] as having found trypanosomes in the anopheles near Calcutta. Not having access to the original paper we are unable to state whether the origin of this infection was established. Another illustration on this point is that given by Durham[24] in his report of the yellow fever expedition to Parà. Some specimens of *Stegomyia fasciata* were placed in a cage with a bat, and when examined later showed trypanosomes. It is probable that these came from the blood of the bat which, however, had not been previously examined. The presence of trypanosomes in bats has been noted by Grassi,[29] Dionisi,[21] Testi,[89] and by Donovan (cited by Laveran and Mesnil[57]).

Finally, to this list of observations bearing upon the presence of trypanosomes in the blood of birds must be added the recent discovery (May, 1904) by Levaditi of a trypanosome in the blood of a Java sparrow (padda or rice-bird, *Padda oryzivora*) purchased in Paris. The trypanosome is evidently rare in these birds, for Laveran[52] examined, at one time, a large number without finding any other parasite than *H. Danilewskyi*. This trypanosome has been designated by Laveran and Mesnil[57] as *Tr. paddæ*, who have also given a very full description of this new organism. Thiroux[90] working under their direction has studied this trypanosome and the results have been recently published. From the illustrations and description given by him we are inclined to consider this as probably identical with the large form of Danilewsky, the trypanosome of Hanna, and the large type found by us. The question of identity, however, can not be settled owing to the absence of a comparison of the cultures of these organisms.

METHODS EMPLOYED.

Before discussing the results obtained in this investigation it may be well to give in detail the methods which we have employed. These, in general, cover the direct detection of trypanosomes in the blood, their cultivation, and the inoculation of birds with such pure cultures.

The necessary drop of blood for the direct examination was usually obtained from the marginal vein on the inner side of the wing. For this purpose the feathers were first removed, and the skin was then washed with a little water. By means of a pair of sharp-pointed scissors the small vein was cut, and a drop or two of blood was thus obtained. The resulting injury is so slight that the smallest bird, such as a sparrow or canary, can be examined every few days during a long period of time. The selection of a small vessel gives just enough blood for the examination, and obviates the excessive bleeding which is likely to occur when a larger blood-vessel is cut.

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In the case of the dead bird, the blood was taken directly from the heart, either by means of a fine Pasteur pipette, or with the aid of a platinum wire, or by direct contact of the cut surface with the cover-glass.

When examining the fresh blood under the cover-glass care was taken to apply just enough pressure so as to obtain a single layer of blood corpuscles. When this is properly done it becomes a very easy matter to detect intra-cellular parasites, even when these are present in very small numbers. This is especially true of the parasites of the red blood cells which are easily recognizable by their hyaline bodies, the "pseudo-vacuolae" of Danilewsky, and by the presence of the blood pigment or melanin. The so-called "leucocytozoa" are less easily recognized because of their resemblance to white blood cells, and the almost total absence of pigment granules. This is particularly true when their number is very small. The formation of microgametes, especially during the morning hours, is very prompt, and may attract attention where otherwise the parasite would be unnoticed.

The detection of trypanosomes in the fresh blood is by no means an easy matter, owing to their extreme scarcity. And yet this method is, as a rule, more delicate than the examination of stained preparations. When, for instance, there is but a single trypanosome in the blood under the cover-glass, it is more likely to attract attention by its movements and the agitation of the blood corpuscles than is the stained preparation in which the organism is, likely as not, concealed by a mass of cells. We have repeatedly been unable to find trypanosomes in stained specimens of blood the direct examination of which had readily revealed their presence.

This statement holds true also for the very small free hemogregarines or vermiculi. Notwithstanding their small size, their peculiar motion, and that of the blood cells with which they come into contact, as well as their characteristic refractile appearance, assists materially in their detection.

The fresh blood preparation should be examined first for the presence of filaria with a low power, such as a No. 3 Leitz objective. These forms, owing to their large size, can be readily detected thus, even when but one or two chance to be present in the specimen. The No. 7 objective is sufficient to show the other organisms, and is especially useful for rapid orientation. Of course, the oil-immersion must be used to establish the nature of doubtful parasites, and to bring out further details.

The method of staining which we used in the early part of our work was essentially that of Romanowsky as modified by Nocht. The solutions employed were:

1. An aqueous one per cent solution of eosin (Höchst).

2. An aqueous one per cent solution of medicinal pure methylene blue (Höchst).

The latter must be "ripened" before use. This is accomplished by adding one-half per cent of crystallized sodium carbonate, and allowing the solution to stand for several days in a paraffin bath at about 60°. A better and more rapid procedure is to place the flask containing the solution in a boiling water-bath for about half an hour, and during this time to pass through the liquid a stream of steam. The latter is generated in a flask from which it is carried into the solution through a drawn-out piece of glass

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tubing. To this product one-half volume of one per cent methylene blue solution is then added.

The actual staining mixture is made by adding to about 10 c.c. of distilled water in an Esmarch or Petri dish three or four drops of the eosin solution. By slight stirring the eosin is distributed through the liquid after which about from two-thirds to one c.c. of the "ripened" or polychrome methylene blue solution is then added, and the whole stirred.

The blood smears, either on cover-glasses or on glass slides, are first fixed by immersion for about 10 minutes in a mixture of equal parts of absolute alcohol and ether. The cover-glasses are then floated on the staining mixture with the specimen side down. When slides are used these are also turned with the specimen side down, but with one end resting on the edge of the tilted Esmarch or Petri dish.

The specimens are allowed to remain in contact with the dye for about 15–20 minutes after which they are rinsed in tap-water and dipped for a few moments in an eosin solution of about one-half of one per cent strength. This serves to remove the excess of methylene blue, and imparts the desired contrast tint of eosin. The preparation is then rinsed and examined in water, after which, if satisfactory, it may be floated off, dried, and mounted in Canada balsam. When properly prepared the specimens will retain their color for several years.

The formation of a dirty deposit on the specimen can be avoided by the use of absolutely clean cover-glasses and by the use of a fresh eosin solution. The latter is prone to deterioration, and hence should be freshly prepared, every week or two. The preparation which is properly stained will show, in the case of bird's blood, the nuclei of the erythrocytes stained a deep red, while the remainder of the cell has a slight eosin tinge. The nuclei of the large leucocytes are stained pink-red while the plasma is blue. The blood plates which are scattered through the specimen appear as irregular discs of a bright red color. The nuclei of the parasites are stained a more or less deep red, while the plasma takes on a blue of variable intensity. It is least stained in the case of proteosoma, slightly more with halteridium, and most strongly with the larger "leucocytozoa." In the case of the latter the plasma of the male cell is stained a pale blue, whereas that of the female cell is usually stained a very deep blue.

The bird trypanosomes do not apparently stain as readily as do those of the rat and of Nagana, for it is the exception to find specimens with well-stained nuclei and flagella. More often the space corresponding to the nucleus is colorless or nearly so. The flagella are usually very indistinct, but nevertheless their presence can often be demonstrated. When the stained preparation fails to show free flagella it must not be assumed that these are absent, for an examination of the fresh living parasite, as found in the blood and especially as present in the culture, will show their presence. At no time have we found trypanosomes with the terminal, free flagella absent.

The staining of cultures is even more difficult than of the blood preparations. Not only is it difficult to stain the nuclei and flagella, but the precipitation of dye upon the cover-glass is such as to make the specimen worthless. It is only recently that with the aid of Mr. Torrey we have been able to modify

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the method of staining so as to obtain satisfactory preparations of this kind. The slides thus prepared have made it possible to secure the excellent photographic reproductions of the cultural trypanosomes shown in the accompanying plates.

The cultivation method, as will be shown later, is far superior to the direct examination, inasmuch as it enables the isolation of trypanosomes when apparently none can be detected by the microscope. The method employed is essentially the same as that which we have used in our work on *Tr. Lewisi*,[64] *Tr. Brucei*,[65 66] and *Tr. Evansi*.[70] In one respect, however, it has received an important modification. It will be remembered that in the work on *Tr. Brucei* only eight per cent of the infected animals gave cultures of this organism. In endeavoring to ascertain the reason for the failure in this large percentage of cases we found that the concentration of the meat extract, as used for the preparation of the agar, was a most important factor. The result of this inquiry has been published by one of us (Dr. MacNeal[60]). The main fact established is that an excess of meat extract inhibits, possibly by overstimulation, the development of the initial culture, whereas a smaller amount favors the growth. This is particularly true of the initial or first generation, but after this is once obtained the organism readily adapts itself to the ordinary medium.

Consequently, we have used in this work an agar prepared according to the following formula:

Extractives of 125 g. of rabbit or beef meat in 1,000 c.c. of distilled water; two per cent Witte's pepton; 0.5 per cent salt; two per cent agar; and 10 c.c. normal sodium carbonate solution.

The agar thus prepared is tubed and sterilized in an autoclave at 110° for 30 minutes. When cooled to about 50°, two volumes of defibrinated rabbit's blood are added, and the mixture is then allowed to solidify in an inclined position. When firmly set it is placed upright for a few minutes until a few drops of water of condensation appear. This liquid is then inoculated with a drop of blood taken from the heart of the bird by means of a drawn-out tube pipette. In some instances, as when large birds are used, the blood may be drawn from the median vein by means of a sterile syringe. The cotton plug of the tube is then cut off short, moistened with mercuric chloride, and the tube is covered with a rubber cap, after which, it is placed at 25° for about a week.

Under these conditions the bird trypanosomes grow readily and even luxuriantly. As already stated the cultures often succeed when there is no microscopical evidence of the presence of the flagellates. This fact in itself shows how easily they adapt themselves to the new medium. It may be safely said that their cultivation is as easy as that of *Tr. Lewisi*. As a rule it is possible to recognize the presence of growing trypanosomes in the tubes on the third day. On the sixth to the seventh day they are usually extremely abundant and very actively motile. The picture presented by such an active vigorous culture is interesting in the extreme, and varies considerably with the species of trypanosome under observation.

It will be seen that the cultivation method not only serves the purpose of demonstrating the presence of trypanosomes, but also furnishes in many cases

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a positive means of differentiating species. This in itself makes it an invaluable addition to the purely morphological method of studying these protozoa. We have heretofore shown that the cultural characteristics of the Nagana, Surra, and rat trypanosomes are very different, and permit of their ready identification. It may be added that the bird trypanosomes, in culture, differ markedly from each of these three organisms as well as among themselves.

When cultivation is attempted it is always advisable to inoculate three or four tubes of the medium for the reason that it may happen that but one out of a set of such tubes may develop. This is readily understood when it is borne in mind that the number of trypanosomes may be very small, scarcely more than one in a drop.

As a routine we have found the following procedure useful: The blood is taken from the heart by means of a sterile pipette and transferred to four tubes of blood agar (one to eight meat extract), which are then capped and set aside at 25°. The remaining blood is used to make cover-glass or slide smears which when air-dried are fixed in a mixture of equal parts of alcohol and ether, after which they are stained by the Romanowsky method. After spreading the films a slide of the fresh blood is examined for hematozoa, first with the No. 3 Leitz objective, then with a No. 7, and finally, if necessary, with the oil-immersion lens. As a rule, this examination was made without the use of a movable stage. However, when examining stained and mounted preparations, it is advisable to make use of one, and for this purpose the new Zeiss model is to be recommended. This, in addition to being detachable, enables one to locate a given field, even when the slide is transferred to another microscope. Although not intended to be used with the microphotographic stand, we have so used it to great advantage.

The injection of the trypanosome cultures can be made either subcutaneously or into the breast muscle. The intraperitoneal injection we found to be rather dangerous, and for that reason we abandoned it in favor of intrapleural injections. These can be made with the greatest of ease and with the least possible injury to the bird. For this purpose the needle of the syringe is inserted obliquely through the furcular angle into the right pleural cavity. Relatively large doses, even one-half c.c., can thus be introduced into a small bird, such as a sparrow or canary. The feathers over the wish bone should be removed and the skin washed previous to making the injection.

UNSUCCESSFUL CULTIVATION EXPERIMENTS.

The immediate object of this investigation was to establish, if possible, the correctness of Schaudinn's views as to the trypanosome stages of the intracellular parasites. For this reason cultures were made, as a rule, from such birds as contained hemocytozoa. In some instances the cultures were made from birds which contained no recognizable parasites. In the case of the infected birds special care was taken to use those which were very rich in fully developed hemocytozoa, that is to say those which

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gave rise to an abundance of microgametes. Nevertheless, such attempts were often fruitless, as will be seen from the synopsis which follows.

It may be stated in advance that of the tabulated 26 negative attempts at obtaining cultures (including those of three canaries), 19 were made with birds very rich in one or more intracellular parasites, such as *proteosoma*, *halteridium*, *H. Rouxii* with free hemogregarines, and *H. MacCallumi*. The remaining six birds had received previous injections of cultures of trypanosomes or other parasites. The failure in such indicates that, in the bird used, the trypanosomes, in the interval which elapsed, had disappeared from the blood. An instance of this kind was afforded by robin No. 270, in the blood of which trypanosomes were found 19 days before the culture was attempted. Only one tube was inoculated, and it failed to give a growth, showing that either the number of parasites had greatly decreased or had entirely disappeared in the interval.

Cultures were also attempted in the case of 10 robins which had a more or less intense infection with one or more of the following: *Pl. Vaughani*, *H. majoris*, *halteridium*, and *filaria*. Owing, however, to contamination with bacteria failure resulted. It is quite probable, judging by the large number of successful cultures obtained from robins, that some of these would have shown trypanosomes.

1. Sparrow, No. 53.—Received an injection of blood and internal organs of robin No. 51 which had numerous *halteridia*, a few *H. majoris*, and a few trypanosomes; died next day. Four tubes were inoculated; result, negative.

2. Sparrow, No. 76.—Received an injection of suspension of blood and organs of wren, No. 86, rich in trypanosomes. Two days later a few *proteosoma* appeared, these increased greatly, and on the fifteenth day, when very rich, it was etherized and four tubes inoculated. Result, negative.

3. Sparrow, No. 75.—Received same injection as preceding; repeated examinations negative. Twenty days later received injection of blood of robin No. 271 rich in *halteridium*, *H. majoris*, *Pl. Vaughani*. No infection; died 10 days after second injection. One tube inoculated, negative.

4. Sparrow, No. 108.—Received injection of blood of hemogregarine sparrow, No. 111. Four days later *proteosoma* were rich. Was etherized on the fifth day and four tubes inoculated; result, negative.

5. Sparrow, No. 113.—This sparrow was rich in *H. Rouxii* and in the long, free hemogregarines. Was etherized and two tubes inoculated; result, negative.

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6. Sparrow, No. 123.—Extremely rich in *proteosoma*. Was etherized and two cultures made; result, negative.

7. Sparrow, No. 129.—Was like the preceding; two tubes inoculated with the heart-blood were negative.

8. Sparrow, No. 140.—Was inoculated with blood of *proteosoma* sparrow. Eleven days later, when extremely rich in segmenting forms, was etherized and four tubes inoculated. Result, negative.

9. Sparrow, No. 149.—Sparrow rich in *halteridium*. Was etherized and two tubes inoculated; result, negative.

10. Sparrow, No. 151.—Received an injection of blood of above *halteridium* sparrow. Eleven days later showed few *halteridia* and many *proteosoma*. Was etherized and two tubes inoculated. Result, negative.

11. Sparrow, No. 180.—Was inoculated with blood of *halteridium* sparrow, No, 149. No infection; died nine days later. One tube inoculated with negative result.

12. Sparrow, No. 181.—Was inoculated the same as preceding, and died also on the ninth day. No infection. One tube was inoculated with negative result.

13. Sparrow, No. 216.—Was extremely rich in all stages of *proteosoma*. Was chloroformed, and four tubes were inoculated. Result, negative.

14. Sparrow, No. 233.—Rich in *proteosoma* with few *halteridia;* was chloroformed and two tubes inoculated. Result, negative.

15. Sparrow, No, 234.—Was rich in *proteosoma* and *halteridia*. Immediately after death four tubes were inoculated with negative result.

16. Sparrow, No. 297.—Was very rich in *proteosoma*. Immediately after death two tubes were inoculated, but with negative result.

17. Sparrow, No. 307.—Was very rich in *halteridia* and in hemogregarines. Was chloroformed and one tube inoculated with negative result.

18. Sparrow, No. 313. Very rich in *proteosoma;* had some free gregarines; was chloroformed and two tubes inoculated. Result, negative.

19. Sparrow, No. 319.—Rich in all forms of *proteosoma* and in *halteridia;* was chloroformed and two tubes inoculated with negative result.

20. Sparrow, No. 338.—Was injected with a citrated suspension of cultures of strains A, B, C, D, E. Seven days later segmenting forms of *proteosoma* were found (latent infection). Died on the fourteenth day. One tube was inoculated with negative result.

21. Sparrow, No. 339.—Injected the same as the preceding; also died on the eleventh day. No infection. One tube was inoculated with negative result.

22. Chipping sparrow, No. 263.—*Halteridia* very abundant. Immediately after death three cultures were made. Result, negative.

23. Mourning dove, No. 85. *H. MacCallumi* numerous. Blood drawn from vein with syringe. Four tubes were inoculated. Result, negative.

24. Canary, No. 1.—Received an injection of trypanosome cultures, strains J. K. L. Four days later was killed by accident. Two tubes were inoculated at once, but with negative result. No infection.

25. Canary, No. 2.—Received the same injection of trypanosome cultures as sparrow No. 20 above. No infection. Was killed sixteen days later and one tube inoculated. Result, negative.

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26. Canary, No. 8.— Was injected with *proteosoma* blood of sparrow. Died ten days later with *proteosoma* very abundant. Three tubes were inoculated with negative result.

The failure to obtain cultures of trypanosomes from birds richly infected with hemocytozoa may be taken to show that these two classes of parasites are entirely distinct. This conclusion will be strengthened by other observations.

RECOGNIZED CASES OF TRYPANOSOMATIC INFECTION.

The following table is a summary of the findings in birds having trypanosomes. These it will be seen comprise 15 species and 38 cases. The second column indicates the result of the microscopical examination of the blood in the fresh condition. A negative result is shown by the sign —. The third column gives the findings in stained preparations of the blood. An ordinary examination of such stains was very often negative and for that reason the slides were examined, field by field, with the aid of a movable stage. In some instances the blood was spread on the slide and such smears were covered with a glass slip 21 × 42 mm. Usually, however, cover-glasses were employed which were about 21 mm. wide. In several cases (as in S and P), three or four slide smears were examined before the one trypanosome was found. Obviously, the detection of trypanosomes in the blood when present in such small numbers is largely a matter of chance.

The fourth column shows the results obtained by the cultivation method. As will be seen at a glance this procedure offers the surest means of detecting the trypanosomes. In only one instance did the method apparently fail, and that was in the case of robin, No. 270, in which the parasite was seen 19 days before the culture was attempted. The dotted sign (.....) in this column means that cultivation was not tried. The letters designate the strains isolated, and the numbers the generations or sub-cultures through which each was carried.

The fifth column gives the name of the species found, based upon the characteristics given later on. A number of these are open to question since the cultural or other characteristics are somewhat different from the type species.

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TABLE I.

SHOWING RESULTS OBTAINED WITH BIRDS HAVING TRYPANOSOMES.

		TRYPANOSOMES DETECTED IN			SPECIES	OTHER PARASITES PRESENT
		Direct	Stain	Cult're		
1. Blackbird, red-winged (*Agelaius phœniceus*)	No. 401	−	+ 1 small 2 large	.....	*Tr. avium*	Filaria only.
2. " rusty (*Scolephagus carolinus*)	No. 420	−	−	A 10	Tr. sp.? Type 4	None.
3. Bluebird (*Sialia sialis*)	No. 426	−	−	B 8	*Tr. avium*	Filaria only.
4. Blue jay (*Cyanocitta cristata*)	No. 244	+	+ 8 small 18 large	K 10	" "	*Hæmoproteus majoris.*
5. " " "	No. 273	−	−	M 2	Tr. sp.? Type 4	None.
6. " " "	No. 278	−	+ 18 small 4 large	R 12	*Tr. avium*	*H. Danilewskyi*, very rich, very few *H. majoris.*
7. " " "	No. 408	−	−	Z 9	" " ? Type 1a	Filaria only.
8. " " "	No. 429	−	−	C' 9	Tr. sp.? Type 4	*H. Rouxi?* Gregarines?
9. Dove, mourning (*Zenaidura macroura*)	No. 5	+	+ 5 small	A 7	*Tr. avium*? Type 1a	*H. Sacharovi*, very abundant.
10. " " " "	No. 6	+	+ 1 "	B 22	" " ? Type 1a	" " " few.
11. Gold-finch, American (*Spinus tristis*)	No. 353	−	+ 1 "	S 10	*Tr. Laverani*	" few; *H. Danilewskyi*, rich.
12. " " " "	No. 354	−	−	X 9	Tr. sp.? Type 4a	None.
13. Flicker (*Colaptus auratus*)	No. 388	−	−	U 10	*Tr. avium*? Type 1a	None.
14. Hawk, red shouldered (*Buteo lineatus*)	No. 350	−	+ 1 large	P 11	*Tr. Mesnili*	*H. Ziemanni*, *H. Danilewskyi.*
15. Oriole, Baltimore (*Icterus galbula*)	No. 41	+	+ 92 small	C 5	*Tr. avium*	None. *H. Rouxi?*
16. " " " "	No. 272	+	+ 8 "	.....	" "	None.
17. Robin (*Merula migratoria*)	No. 46	−	−	AB 4	" "	*H. majoris*, very few.
18. " " "	No. 50	−	+ 2 small	G 4	" "	*Pl. Vaughani*; few *H. Danilewskyi.*
19. " " "	No. 51	+	+ 9 "	D 5	" "	*H. majoris*, rich in *H. Dan'l'skyi.*
20. " " "	No. 53	+	+ 1 "	E 4	" "	" " " " "
21. " " "	No. 54	−	+ 9 "	F 1	" "	" " " " "
22. " " "	No. 184	−	−	I 2	" "	" " few.
23. " " "	No. 214	+ 3 large	−	L 2	" "	" " "
24. " " "	No. 242	+ 2 large	−	J 2	" "	" " "
25. " " "	No. 257	−	−	Q 12	" "	" " "
26. " " "	No. 270	+	+ 1 small 1 large	− ?	" "	" " " Culture attempted 19 days after tr. were seen.
27. " " "	No. 284	−	−	N 2	" "	*H. Danilewskyi*, few.
28. " " "	No. 345	−	−	O 1	" "	None.
29. " " "	No. 376	−	−	V 12	" "	*H. Danilewskyi*, very few; filaria.
30. " " "	No. 393	−	−	W 9	" "	" " *Pl. Vaughani.*
31. Sparrow, English (*Passer domesticus*)	No. 109	+ 1 large	−	.....	" "	None.
32. " " " "	No. 279	+ 2 large	−	.....	" "	None.
33. " " " "	No. 286	+ 1 large	−	.....	" "	None.
34. Song sparrow (*Melospiza fasciata*)	No. 142	−	+ 2	H 4	" "	*Pl. relictum*, very rich.
35. " " " "	No. 288	+	+ 28 small 1 large	.....	" "	*H. Danilewskyi*, rich.
36. Brown thrasher (*Harporhynchus rufus*)	No. 386	+	−	T 8	Tr. sp.? Type 3a	Filaria.
37. Woodpecker, hairy (*Dryobates villosus*)	No. 391	−	+	.....	Tr. sp.?	None.
38. House wren (*Troglodytes aedon*)	No. 86	+ 10	−	.....	Tr. sp.?	None.
Microscopical detection failed entirely in 14; these recognized by culture		16	17	29		15 without intracellular parasites

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The last column gives a list of the other parasites present in each bird. Several of the names are of new species which will be described in the next paper.

The above table embraces the results obtained from an examination of 431 birds, representing 40 species. Of this number 38 (16 species) or 8.8 per cent were found to contain trypanosomes. This figure must not be taken to indicate the actual number of birds infected with these organisms for the reason that cultures were not attempted in all cases.

The actual number of cultivation experiments (free from contamination) made with wild birds, as seen from this table and the preceding summary, is only 53. Twenty-four of these were negative and 29 were positive. Of the cultural attempts, then, 55 per cent were successful. In eight other birds trypanosomes were found with the microscope, and, if at the time an attempt at cultivation had been made, judging from the success under like conditions, the cultures would probably have developed, in which case 37 out of 61 would have been positive, or about 60 per cent.

This statement may be somewhat misleading, for the reason that of the 61 birds in question, 18 were known, from the microscopical examination made at the time, to have trypanosomes. If, therefore, these are deducted, it will be seen that in the 43 birds in which the original microscopical examination failed to show trypanosomes the cultural method revealed their presence 19 times, or in about 44 per cent.

It is evident from these considerations that the percentage of birds infected with trypanosomes is much higher than would be indicated by the limited findings given in the table. It is probable that the careful application of the cultural method to a large series of birds will show that fully one-third, if not more, harbor these parasites.

It is certainly remarkable that the microscope, even after most careful re-examination, should show trypanosomes in only 24 out of 431 birds, whereas the cultural method applied in only 53 cases, excluding attempts in which contamination occurred, should give 29 positive results.

The relation of the intracellular parasites to the trypanosomes

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is of especial interest. In connection with the summary of the unsuccessful cultivation trials it was pointed out that, although cytozoa were very abundant in 19 of those cases, yet no culture was obtained. By contrast the above table shows that trypanosomes may be present, at times in very appreciable numbers, unaccompanied by intracellular parasites. Cytozoa were absent in 15 and present in 23 of the birds known to have trypanosomes.

The existence of a latent infection with cytozoa in these 15 cases is possible, but even with that assumption it is difficult to reconcile this finding with the supposed relation of trypanosomes to this class of organisms. Moreover, it can be shown that birds may harbor trypanosomes for weeks and months without showing any infection with intracellular parasites.

In one instance, in particular, a canary developed trypanosomes in its blood four days after an injection of a culture of strain D. The trypanosomes were found at times, though not always, during the following 11 weeks, when the bird died, but at no time was there any indication of the presence of a cytozoön.

Thiroux,[90] the only one previous to ourselves who has succeeded in infecting birds with trypanosomes, found that the period of incubation ranged from a few (12) hours to 18 days. This variation was due, in part, to the natural resistance of the bird (padda), and, in part, to the method of infection. The inoculations were subcutaneous, intramuscular, intravenous, or intraperitoneal; the latter being the surest method. The infection was of variable intensity, in some cases the parasites being scarce, while in others they were very numerous. Indeed, one of the padda birds died apparently from the severe infection which developed. In the case of moderate infection, usually following a short period of incubation, the trypanosomes increased in numbers during a period of 9 to 15 days after which they decreased and finally remained stationary, in some instances during a period of 40 days. In addition to the padda, five other species of birds, including the green and common canary, were successfully infected.

The significant feature of Thiroux's experiments is that, notwithstanding this rich experience in the artificial infection of birds with trypanosomes, no mention is made of the appearance of

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intracellular parasites which it is reasonable to expect would appear if the latter were but stages in the life-history of the former. The fact that no mention is made of the presence of cytozoa leads us to believe that they were not found. With this assumption, his results are in accord with ours on the canary mentioned above. They agree furthermore with the observed fact noted in the table that trypanosomatic infection of wild birds may exist without association with intracellular parasites.

Furthermore the study of the trypanosomes found in the blood, and also of those obtained by cultivation, shows that there are several distinct species which exhibit no constant association with a given cytozoön. Thus, the most common species, described further on as *Tr. avium*, was associated with *H. Sacharovi*, *H. majoris*, *H. Danilewskyi*, *H. Rouxi*, *Pl. Vaughani*, or with *filaria* in addition to its very frequent single occurrence. Under these circumstances it would indeed be difficult to establish a relationship with any of the intracellular organisms mentioned.

MORPHOLOGY OF TRYPANOSOMES IN BLOOD.

Observations made upon the trypanosomes found in the fresh blood or in stained preparations are insufficient for the purpose of identification. As in the case of bacteria, the cultural characteristics and the animal experiment must, so far as possible, be utilized in order to differentiate allied organisms. In the study of the trypanosomes of mammals it has been, after all, the animal experiment which has served the purpose of identification, since the morphological variations are at times so slight as to be almost negligible. In three of these infections the cultural method has supplied an additional means of recognition. Without doubt, the number of species of trypanosomes met with in mammals and man is very large. Moreover, with perfected methods it will be found that a given species of mammal may be subject to natural infection with several kinds of trypanosomes, some of which may be very pathogenic, while others are not. In other words infections of this type will be found to parallel those of other protozoa and even of bacteria. It will be sufficient to take a single illustration—that of malaria. Malarial infection, as met with in man,

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monkeys, bats, and birds, is due to different species of parasites. And, in the same species of animal, as man or bird, several species of these parasites may be found. That it is possible for one species of bird to harbor two kinds of trypanosomes will be shown farther on.

As indicated above, a thorough study of a given trypanosome embraces observations upon the living and stained organism as found in the blood, an examination of its cultural characteristics, and a determination of the pathogenic action of the pure cultures upon the same species as the host, as well as upon other species. It has not been possible to meet all these requirements in every case of trypanosome infection in the present investigation for reasons which will be readily seen.

In the 24 cases recognized by means of the microscope, the trypanosomes were found in the living condition in the blood only 16 times. The stained preparations showed them to be present in 17 cases. In 14 of the birds the examination of the fresh and stained blood was negative, the organisms being detected solely by the cultural method. It should perhaps be stated that the number of positive stains would have been much less than that given above were it not for the very valuable check afforded by the cultivation process. It happened several times that the ordinary examination of the stain was negative, but inasmuch as the culture eventually developed it led to a re-examination of the slides, field by field, and in a few instances the laborious search was rewarded by finding the trypanosomes in the stains. In the case of culture S (*Tr. Laverani*) only one trypanosome could be detected thus on three slides made from the original blood. Similarly, only one could be found on four slides of the blood which gave culture P (*Tr. Mesnili*).

The difficulty of detecting trypanosomes in stained preparations can best be shown in the case of a canary which was artificially infected by means of culture D. During a period of 11 weeks the parasites were repeatedly found in living condition in the blood, and, although stains were made at the same time, they were invariably negative. When the bird died trypanosomes were found as usual, but, although seven slide smears

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were made and carefully examined on a movable stage, the examination consuming about 20 hours, the result was the same as before.

With but few exceptions, the trypanosomes which we found in the living state in the blood were one of two types, corresponding very closely to the *Tr. majus* and *Tr. minus* of Danilewsky, so much so that we are obliged to consider our common forms as identical with the ones described by him. Moreover, we are in accord with Danilewsky in regarding these two forms, notwithstanding their great difference in size, as belonging to one and the same species—*Tr. avium.* This statement, however, refers only to the common trypanosomes of birds since, as will be seen, there are several species.

In five of the birds, as can be seen from the table, the large and small forms were found together. At first sight, it would appear as if the two forms represented distinct species, but that such is not the case is readily seen from the cultural characteristics. In other words, the cultures made from the blood of birds having only the small form, or only the large form, or both forms at the same time, present exactly the same appearance. The position of the blepharoplast with reference to the nucleus clearly shows that the large form is to be considered as preparing for division. Furthermore, the presence of this type in very young birds indicates that the infection is of recent origin. In this respect it corresponds to the large form of *Tr. Lewisi* which appears in the blood on about the sixth to the ninth day of infection. As is well known, in the rat after the stage of active multiplication of *Tr. Lewisi* is passed, the large form disappears, and only the ordinary or typical form persists. That there is a close generic relation between *Tr. Lewisi* and *Tr. avium* is seen in the marked similarity of the cultures, both giving rise to actively motile free-swimming forms, and to characteristic multiplication rosettes, the individuals composing which have their flagella directed toward the center.

In view of these facts we are led to regard this large form as a multiplication, or possibly sexual type, of the smaller and more common form.

This large form usually appears as an S-shaped body having

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a well-developed, undulating membrane, which terminates in a free flagellum 10–15 μ long. The body proper varies in length from 21–30 μ, but at times it may attain even 40 μ and more. The posterior end for about 12–15 μ is narrow and tapers to a point. It shows a peculiar stiffness, due without doubt to the fact that the blepharoplast is near the nucleus. The nucleus is readily visible in the living form as a large round body, or, more often, as an oval which is placed transversely across the body, filling out the entire width. The width of the cell opposite the nucleus is from 5–7 μ. As might be expected, owing to its large size, this form shows very little tendency to travel out of the field of the microscope, and for that reason can be readily kept under observation for hours. The contortions, however, are very active, and the organism is constantly changing from side to side, at times straightening out, or forming a coil. For that reason the measurements as given above, made on the living parasite, are only approximate.

The stained preparation shows the large form in the characteristic S-shape (Plates 2 and 3) or in the circular position (Plates 3 and 4). The centrosome, it will be seen, lies close to the nucleus and is in a large colorless space. The nucleus itself does not stain readily, and for that reason appears as a large light body in the middle of the cell. Beautiful striæ or myonema lines, six or eight in number, are easily made out on the part overlying the nucleus. These lines are continued the entire length of the cell. The long stiff or atrophied posterior end is very noticeable. The undulating membrane is visible as a delicate, fairly wide, and rather wavy border on one side of the organism. As will be seen from the table, the length of the body of the large form varies within wide limits, that is from 35–65 μ. The whip is probably 15–20 μ long.

The two forms described represent the common types as met with in the blood of birds, and are to be regarded as belonging to the same species. Two other trypanosomes, however, were found in the blood.

The first of these met with, in the blood of a hawk, is shown in Fig. 1, Plate 5. The large size (see culture P, Table 2) and peculiar shape mark it at once as distinct from the preceding.

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Moreover, the cultural characteristics are totally different and stamp it as a new species. We have named it *Tr. Mesnili* in honor of Dr. Mesnil, of the Pasteur Institute. The full description of this form will be found later on.

The second trypanosome, referred to above, was found but once in the stains made from the blood of a goldfinch. It is designated as Culture S in the table, and from the measurements there given it will be seen to correspond closely to the ordinary small form of *Tr. avium*. Indeed, if the size was the only criterion, there would be little hesitation in regarding it as being of the same species. The presence of a large number of granules in the posterior half would hardly be sufficient evidence for separating it as a distinct species. And yet the cultural features of this organism are unlike those of *Tr. avium* or of *Tr. Mesnili*, and on that account it must be considered as a new species. We have designated this organism as *Tr. Laverani* in honor of Dr. Laveran.

The small form is the most common one met with in the blood of birds. The total length, whip included, is about 25–30 μ, or a trifle more than the length of two blood corpuscles. The body proper is about 20 μ long, though exceptionally it may vary from 14 to 25 μ. The width ranges from 3.5–5 μ. The nucleus, of nearly the same width as the cell, is at times visible, but not always. The posterior half of the body usually shows minute granules or globules, but at no time is there any indication of pigment granules. The body is usually in the form of a straight spindle, widest in the middle, tapering quite evenly in both directions. The anterior end is usually bent at right angles to the line of the body. There is an absence of the stiff posterior end as met with in the larger form. The undulating membrane is easily made out, though it is not as wide as in the other type. It starts very close to the posterior end, and as it passes forward, crosses the body, forming one or two wavy bends. It finally terminates in a free flagellum, 8–12 μ long. The motion is sluggish, and, as in the other form, there is but little tendency to travel out of the field of view.

The small form as seen from the photographs (Plates 1–4) is a spindle-shaped body which tapers to a sharp posterior end.

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The large centrosome or blepharoplast at times appears to be at the very tip, while again it may be four or five μ distant. It is often surrounded by a colorless or achromic zone. The flagellum, in several of the preparations, is seen to start from the centrosome and form the outer border of the undulating membrane, eventually becoming the free whip, which is about 8–10 μ in length. The nucleus is not always distinct, but it can often be seen as a large body nearly as wide as the cell. In the preparations which have become crushed or flattened out (see Fig. 4, Plate 1, and Fig. 2, Plate 3) the nucleus and border of the undulating membrane can be seen very plainly. In a few instances a faint suggestion of striæ on the surface of the body can be made out.

The description and measurements of the two forms as given above, unless otherwise indicated, refer to the organisms as found in the living condition. Obviously, it is not possible on account of the constant movement to make very accurate measurements, whereas with the stained preparation this can be done very satisfactorily. The stain has the further advantage in that structural differences are readily brought out, if any exist.

The measurements given in the appended table include all the kinds of trypanosomes met with in the stained preparations. In the few instances where the number of organisms was large, only five or six were taken for this purpose.

The small forms are grouped together in order to show the close similarity in size. At first sight it would seem as if there were two types, one of which was about 20 μ in length with the centrosome close to the posterior end, usually not over 1 μ from the tip; while the other was about 25 μ in length with the centrosome about 5 μ from the end. In view of the fact, however, that the cultural features are so closely alike, if not identical, it has not seemed desirable at present to place too much stress on this slight difference, especially as a still greater variation is met with in the case of the large form which, as mentioned above, must be regarded as belonging to the same organism. It is quite probable the larger of the small forms, with its centrosome at a distance from the tip, constitutes a transition to the large form which has its centrosome close to the nucleus.

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TABLE II.

GIVING MEASUREMENTS OF TRYPANOSOMES AS FOUND IN STAINS.

Trypanosomes Found in		Length of Free Flagellum	Length of Body	Distance from Centrosome to Posterior End	Distance from Centrosome to Anterior End	Distance from Centrosome to Center of Nucleus	Width of Body at Nucleus	Size of Nucleus	Length of Centrosome
Baltimore oriole, No. 41, culture C.	*a*..	8.0	21	1.0	20	10	5.0	3×5	0.7
	b..	....	20	0.5	19.5	10	5.0	4×3	0.7
	c..	....	20	1.0	19	12	5.0	5×4	0.7
	d..	....	21	0.5	20.5	10	4	4×3	0.7
Robin, No. 53, culture E.	*a*..	....	20	0.5	19.5	11	5.0	2.5×3	1.0
Robin, No. 270, No culture. Small form.	*a*..	8.0	20	0.5	19.5	12	5.0	4×4	1.0
Blue jay, No. 244, culture K. Small form.	*a*..	10	18	1.0	18	11.5	5.0	4×3	1.0
	b..	....	19	1.5	17.5	8.5	5.5	5×3	0.5*
	c..	....	16	1.5	14.5	9.5	5.2	5×3	0.7*
	d..	....	18	1.0	17.0	9.0	4.0	4×2	1.0
	e..	....	19	1.0	18.0	9.0	4.0	3×2.5	1.0
	f..	....	18	1.5	16.5	9.5	7.0	6×3	1.0*
Goldfinch, No. 353, culture S. A distinct species.	*a*..	....	20	1.0	18.0	8.	6.0	4×2	0.6
Song sparrow, No. 142, culture H.	*a*..	9.0	20.0	1.5	18.5	8.5	4.5	4×3	0.6
	b..	....	19.0	1.5	17.5	9.5	9.0	6×3	0.7*
Mourning dove, No. 5, culture A.	*a*..	6–8	22	1.0	21.0	10.0	5.0	4×4	1.0
	b..	....	23	1.0	22.0	10.5	5	4×5	0.7
	c..	....	22	1.0	21	9.5	4.0	3×4	0.7
	d..	8	22	1.0	21	9.0	4.5	3×4	0.5
	e..	....	24	1.0	23	10.0	4.5	4×4	0.7
Mourning dove, No. 6, culture B.	*a*..	8–10	24	1.0	23	10.0	5.0	4×4	0.7
Robin, No. 50, culture G.	*a*..	10	16	1.5	14.5	6.5	6.0	3×5	0.7*
	b..	9–10	23.5	5.0	18.5	6.5	6.0	3×5	0.5
Robin, No. 51, culture D.	*a*..	5.5+	23	4.0	19	12	5.0	4×5	0.7
	b..	9.0	25	5.0	20	10	5.0	4×5	0.7
	c..	....	25	5.0	20	10	5.0	4×5	1.0
	d..	....	25	5.0	20	10	5.0	4×5	1.0
	e..	....	24	5.0	19	9+	4.0	....	0.7
Blue jay, No. 278, culture R. Small form.	*a*..	9.0	24	6.0	18	7.0	5.0	4×4	0.5
	b..	13	25	5.0	20	7.0	5.0	4×5	0.5
	c..	8.0	24	5.0	19	8.0	5.5	4×5	0.5
	d..	....	24	5.0	19	7.5	5.0	3×4.5	0.5
	e..	7	24	5.5	18.5	8.5	5.0	3×5	0.5

* Indicates that the specimen was somewhat crushed or flattened. As a result it is shorter and wider than usual.

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TABLE II—*Continued.*

Trypanosomes Found in		Length of Free Flagellum	Length of Body	Distance from Centrosome to Posterior End	Distance from Centrosome to Anterior End	Distance from Centrosome to Center of Nucleus	Width of Body at Nucleus	Size of Nucleus	Length of Centrosome
Robin, No. 54, culture F.	*a*..	....	20	4.0	16	8.0	5.5	2×5	1.0
	b..	8.0	21	4.0	17	7.5	5.0	3×4	1.0
	c..	....	20	3.5	16.5	8.0	5.5	3×5	0.7
	d..	....	20	4.0	16	7.5	4.0	2×3	1.0
	e..	....	22	4.0	18	8.5	5.0	3×5	1.0
Song sparrow, No. 288, No culture. Small form	*a*..	8+	18	2.0	16	7.0	5.0	4×4	1.0
	b..	....	17	4.0	13.0	6.0	4.0	3×3	0.7
	c..	....	19	3.0	15.0	10	3.5	3×3	0.5
	d..	....	17	2.0	15.0	8.0	4.0	3×3	1.0
	e..	....	20	3.0	17	8.0	4.0	3×3	0.7
	f..	....	21	1.5	19.5	10.0	5.0	4×4	1.0
Baltimore oriole, No. 272, No culture.	*a*..	10	20	4.0	16.0	7.0	5.0	2.5×2	0.5
	b..	8	20	5.0	15	8.0	6.0	2.5×3	0.5
	c..	6.0+	21	5.0	16	8.0	4.0	2×3	0.5
	d..	10	21	5.0	16	8.0	4.0	2×3	0.5
	e..	8	17	3.0	14	6.0	7.5	3×5	0.5*
	f..	7+	20	5.0	15	9.0	7.0	3×5	0.7*
	g..	7	22	6.0	16	8.0	6.0	3×5	0.7*
	h..	7	21	2.0	19	9.0	6.5	3×3	0.7
Blackbird, No. 401, No culture.	*a*..	....	35	15	20	6	4.0	3×3	1.0
	b..	....	35	15	20	6	5.0	5×5	0.7
Song sparrow, No. 288, No culture. Large form	*a*..	....	40	15.0	25	7.0	4.0	4×4	0.7
Blue jay, No. 278, culture R. Large form.	*a*..	....	53	19.0	34	7.0	5.0	4×5	0.5
	b..	20.0	50	17.5	32.5	6.5	6.5	5×5	0.5*
	c..	*Ca.*10	49	13.0	36	10	6.5	5×5	0.5
	d..	12	52	19.0	33.0	7.0	5.0	4×5	0.5
Blue jay, No. 244, culture K. Large form.	*a*..	....	50	20	30	7.0	5.0	4×5	1.0
	b..	10+	48	18	30	7.0	5.0	4×5	1.0
	c..	16.0	57	25	32	7.0	6.0	4×5	1.0
	d..	15 0	53	23	30	7.0	5.0	4×5	1.0
	e..	10–15	53	24	29	7.0	5.0	4×5	1.0
Robin, No. 270, No culture. Large form.	*a*..	....	65	20	45	7.5	5.0	5×5	1.0
Hawk, No. 350, culture P. A distinct species.	*a*..	....	50	7.0	43	11.0	8.0	2×3	1.0

*Indicates that the specimen was somewhat crushed or flattened. As a result it is shorter and wider than usual.

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CULTURAL CHARACTERISTICS OF THE TRYPANOSOMES STUDIED.

Very little difficulty is experienced in cultivating the bird trypanosomes. As a rule they grow quite rapidly, so that at 25° their numbers are quite appreciable on the third day. They reach their maximum on about the seventh or eighth day after which they give rise to spherical involution forms which soon agglutinate or gather into large masses. These become coarsely granular, show highly refractive bodies, and eventually break down into a mass of granular débris. Living forms are rarely to be seen after about two weeks, although, exceptionally, we have met with them at the end of a month. When grown at room temperature, the culture naturally comes on more slowly and remains alive for a much longer period.

Of the 29 strains obtained from that number of birds, only two (strains S and T) showed a much slower growth. In these the trypanosomes were not appreciable until on about the sixth, and did not reach their maximum until about the tenth day.

In all cases the cultures when fully developed were enormously rich in the flagellates. At times there were observed on the surface of the blood agar, just above the fluid, circular colonies, two to three mm. in diameter, which were suspected to be due to bacterial contamination. An examination, however, of such colonies showed them to be a solid mass of actively wriggling trypanosomes.

Subcultures were obtained from all but two (strains F and O) which died out through oversight. In a number of instances these cultures have been kept up for over six months, during which time they have passed through more than 20 generations. Most of the strains have been kept up continuously for the past four months and continue to maintain their original characteristics. A number accidentally died out on account of delayed transplantation.

An examination of the 29 strains in the living condition shows at a glance that they comprise several distinct types, and it may be as well to speak of these for the present by that designation.

The division into types is based upon the characteristics shown by the rosettes and by the free-swimming forms. The main points in this grouping of the cultures can be summarized thus:

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Type 1.—Rosettes and free forms common. The latter are very long and narrow, mere threads, without any noticeable enlargement of the body (*spirochetes*). Growth rapid. In these the blepharoplast is posterior to the nucleus. This is the most common type and corresponds to *Tr. avium.*

Sub-type 1a.—This is much like the preceding, but the spirochetes are shorter and are distinctly wider near the anterior end. They remain, however, long and narrow. Represented by strains A, B, U, and Z.

Type 2.—Large rosettes, the cells being of considerable size and having long central whips. The free forms are likewise large and wide, and show globules. Growth rapid. Represented by strain P—*Tr. Mesnili.*

Type 3.—Rosettes much less common and smaller. The free cells show relatively but little motion, have a blunt posterior end, and the contents are largely made up of big globules. Growth very slow. The cells show a posterior terminal rod. Represented by strain S—*Tr. Laverani.*

Sub-type 3a.—Growth equally slow as preceding. The free cells have a tapering posterior end. Represented by strain T.

Type 4.—Rosettes are very scarce, the free forms predominating. The anterior end of body tapers out gradually along the whip; the cell tapers also posteriorly, contains very small granules, and is actively motile. Rapid growth. Represented by strains M, A′, and C′.

Sub-type 4a.—This is represented by strain X. The rosettes are even more scarce. The growth is rapid and rich. The blepharoplast is very large.

Type 1.—This will be described as *Tr. avium* since it represents the most common form met with in birds. It was present in 18 out of the 29 cultures. In seven birds from which cultures, however, were not obtained the trypanosomes presented the characteristics of *Tr. avium.* It appears therefore that this species was present in 25 out of 38 birds. The particular birds in which this species was found are indicated in Table I.

Two forms are met with in cultures of this type: (1) rosettes of round, oval, or spindle-shaped cells; (2) extremely slender, long, wavy, darting forms. These will be described as "*spirochetes*," since they correspond exactly to what Schaudinn has designated by that name in connection with his work upon the "leucocytozoön" *H. Ziemanni.* The relative abundance of the two forms varies somewhat in the different strains. Thus, in some the rosette form is very abundant from the start, and the spirochetes are rather scarce, whereas in others the latter appear first and in large numbers, while the rosettes are few and small. Again, in some strains, the spirochetes appear to be much longer than in others. These differences we are inclined to look upon

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at present as variations in the several strains rather than as indicative of separate species.

Rosettes.— (See Plates 8 and 9.) In the early stage the rosette consists of a small number of round bodies which may show a few minute granules. They are about 5 μ in diameter and show very little or no motion. They increase rapidly in numbers by means of longitudinal division, giving rise to aggregates of hundreds of cells. Frequently rosettes are met with which, in the somewhat flattened condition due to the pressure of the cover-glass, fill the entire field of a No. 7 Leitz objective.

Eventually, the round or pear-shaped bodies elongate to form ovals, and finally spindle-shaped bodies. At this stage the individuals may be seen to possess a slight swaying motion. The smaller rosettes, consisting of 10–20 cells, measure 14–20 μ in diameter. The spindle-shaped cells are about 10 μ long and about 3 μ wide. They do not apparently break loose from the group, but remain attached. When the growth has reached its full age the spindles begin to form the spherical involutions mentioned above.

It will be seen that the ordinary rosette corresponds in diameter to twice the length of the spindle-shaped cell, that is to say, about 20 μ. The very large rosettes really consist of a number of such smaller ones which can be easily made out in the living condition. The latter may be spoken of as simple or primary and the former as multiple rosettes.

Preparations stained by the Romanowsky method reveal details which cannot be satisfactorily established otherwise. In the first place, the flagella, as in the case of the rosettes of *Tr. Lewisi*, are found to be directed centrally. The relatively large blepharoplast, in an achromic area, is by the side of, or anterior to, the nucleus. This necessarily means that the undulating membrane is very short or even rudimentary, and this explains the absence of motion in the round or oval forms and the slight motion seen in the more elongated bodies. It would appear from this fact that the active motility of the free trypanosome is due largely, if not wholly, to the undulating membrane, and that the free flagellum is incapable of moving or propelling the body.

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Furthermore, the stained preparation throws some light upon the formation of the rosette. It appears that they originate in much the same way as the segmentation form of *Tr. Lewisi* in the blood, described by MacNeal[61] and others. In very young cultures it can be seen that the large, free, spindle-shaped trypanosome shortens to form an oval or pear-shaped body, while at the same time the whip is retracted or absorbed (See Plate 9.) The blepharoplast and the nucleus then divide, giving rise to a pear-shaped body with two nuclei and two blepharoplasts. The cell itself may then divide longitudinally, forming two small cells; or, the division of the nuclei and blepharoplasts may be repeated, resulting in a cell with four each of these bodies. In the case where two cells form by the division of the original, they remain attached for some reason by their rudimentary whips, and, as the process of division is repeated, eventually the typical rosette forms. These groups therefore originate by the consecutive division *in situ* of a single cell, and for that reason the multiplication rosette must not be confounded with agglutination masses. The young forms resulting from the division of the oval cell, especially when the consecutive division is rapid, are quite small, about 5 μ long and $2\frac{1}{2}$ μ wide.

The content of the spindle-shaped cells comprising the rosette stains a deeper blue than does that of the spirochete form. The nucleus is always round, large, and shows eight chromosomes. At times a few small colorless granules and globules are present in the cell. These, however, are insignificant in size when compared with similar bodies found in *Tr. Brucei*, *Tr. Mesnili*, and the other types mentioned later.

Spirochetes.—(See Plates 10 and 11.) These are extremely interesting on account of their very rapid motion and their delicate appearance. As seen in the living preparation they appear as long, slender threads, straight or wavy in outline. They are ordinarily single and dart through the field at great speed. Usually, a very fine whip may be seen at one end, but, at times, this may be so delicate as to be invisible. They frequently attach themselves by means of their whip to red blood cells, which are then rapidly pushed through the field. The spiro-

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chete, as it travels with the whip foremost, may suddenly stop and move backwards for a short distance.

Dividing forms in the young culture can be observed. The division takes place while the cell is in active motion and without any shortening or rounding up as in the case of the rosette type. The stained preparations are especially useful to bring out this condition. The two young cells resulting from division may remain attached for some time at the posterior end, and, in that case, may simulate an agglutinated pair. The two cells, however, are not in a straight line, but form an acute angle, or they may twist about each other in a spiral manner. Each of the cells, while still attached, may undergo division, thus giving rise to a four-celled group.

The spirochetes show a marked tendency to agglutinate. This begins with two cells sticking together at their posterior ends which slightly overlap. (See Figs. 3 and 4, Plate 10.) In the living preparation the line of junction can not be made out, and as a result the double cell appears to be a single organism with one whip at each end.

A third cell may attach itself by its posterior end to the agglutinated pair, in which case the three bodies may be fairly equidistant. Finally more cells come in and join in the same way, eventually giving rise to tangles of hundreds and even thousands of cells (Fig. 5, Plate 11). We have observed masses of these slender, writhing forms fill the entire field of a No. 7 objective. In these groups or masses the whips are always on the outside or periphery, whereas in the rosettes described above the whips are always on the inside.

It will be seen that the two kinds of groups met with in cultures of this organism are of entirely different origin. The multiplication rosette, with its whips directed centrally, arises by the consecutive division of its cells *in situ*, and is in nowise to be looked upon as an agglutination. On the other hand the spirochetes are normally free and divide while in that condition. These free cells agglutinate in some cultures more readily than in others, in which case the cells join at their posterior ends. The very large rosette always shows the constituent groups of

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which it is composed, whereas the agglutinated mass of spirochetes, is without any such regularity.

As the cultures age the spirochetes lose their even form and show a globular enlargement at the posterior end or near the middle. This spherulation may continue until the long form is replaced by a small round body which usually is indicative of the death of the cell. Death may, however, occur without this transformation being completed.

The width of the spirochete in the living condition is about 0.5 μ. The stained preparation, perhaps as a result of flattening, shows the width to be about 1.0 μ. The average length of the spirochete is about 30 μ, but it is not uncommon to meet with cells 50 and even 60 μ long (Plate 10, Fig. 2). The body tapers at each end to a sharp point.

The stain shows that the free flagellum is relatively very short since it measures only about six μ. The blepharoplast, which stains more readily than either the nucleus or whip, is a small dot about 0.5 μ in size. Unlike that of the spindles of the rosette form, it is situated between the nucleus and the posterior end, and distant from the latter by about one-third the length of the body. That is to say, with the body length of 30 μ the blepharoplast is 10 μ from the posterior end. Consequently, the undulating membrane extends along two-thirds the length of this slender body. This explains the extreme motility of this type. Moreover, the shortness of the free whip indicates that it has very little to do with the motion of the cell, as has been pointed out in connection with the rosette form. The position of the blepharoplast, posterior to the nucleus, is an important characteristic, and is not seen in the other species.

The nucleus lies in the anterior half of the body and is approximately of the same width as the cell. In the average spirochete it measures 1×3 μ, while in the very long ones it may be 1×5 μ. When the cell is about to divide the blepharoplast approaches and almost touches the nucleus. At the same time the nucleus becomes round and the cell widens somewhat.

The contents of the spirochete are usually perfectly homogeneous, and in the stains only a few minute granules can be made out.

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Owing to the marked difference between the rosette and spirochete forms it may be supposed that they represent two distinct species. This view, however, is untenable since these two forms are present in every one of the 18 strains. Moreover, at no time have we found a culture which had either form by itself, as could well be expected, if they were distinct species. Again, *Tr. Lewisi* in culture presents essentially the same two types. It has multiplication rosettes and free-swimming forms corresponding to the spirochetes, though much wider. This is the case also with several of the other species, as will be shown presently. In our opinion there can be no doubt but that the two forms belong to one species.

It will be remembered that the blood of the birds from which this type was obtained had, in several instances, two forms of trypanosomes—the small spindle—and the long S-shaped trypanosomes. The question naturally arises as to what relation exists between the two forms in the blood and the two forms in the cultures. It is possible that the large S-form is the source of the rosette and that these represent the female cell, while on the other hand the spindle-shaped trypanosome of the blood gives rise to the spirochete which may be considered as an indifferent, or asexual, form. While the latter are capable of multiplying rapidly, they probably do not change into the other type. On the other hand it would seem as if some of the rosette cells could differentiate into the free form. Such a transformation, however, has not been observed.

Sub-type 1a. This can be easily confounded with the preceding. A close examination however, shows that there are some marked differences. The rosettes appear to predominate, and by their number and size attract attention much as they do in the case of *Tr. Lewisi*. The free-swimming forms are much shorter than those of the preceding type, and are distinctly wider near the anterior end. This gives them a slight tapering appearance. They are very actively motile, have clear contents, and agglutinate by their posterior ends. They may be regarded as intermediate between Type 1 and the following, especially Type 4. This type was met with in strains A, B, U, and Z.

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Type 2. This was met with but once and then in the blood of a hawk. We have named this species, as already stated, *Tr. Mesnili.* Not only is the trypanosome as found in the blood different from *Tr. avium*, but an even greater difference can be seen in the cultures.

Cultures made from the heart-blood showed on the third day several groups of spherical or oval bodies which measured five to eight μ in diameter. They contained a hyaline plasma, somewhat vacuolated and very highly granular, with numerous bright refractile globules of a greenish tint. These globules were about 0.5 μ wide. The cells composing this group showed a long free flagellum on the outside of the mass. A wide, slowly waving, undulating membrane could also be seen. In addition to this group a number of free, motile, coarsely granular trypanosomes, about 16 μ long, were observed.

On the sixth day the culture was fairly rich, and presented a very striking appearance. The free, swarming forms were numerous, and traveled about very rapidly, whip foremost, at times rolling on their long axis. The body of the cell was from 18–21 μ long and about three μ wide. The greatest width was near the anterior end which narrowed rather abruptly and terminated in a long free whip, From the point of greatest width the body tapered gradually toward the posterior end which was either pointed or slightly blunt. Numerous bright globules or refractive bodies filled the posterior two-thirds of the body. Large, rounded, rolling forms are also common.

The actively motile, free forms showed a tendency to agglutinate in a manner highly suggestive of *Tr. Brucei.* That is to say, the cells adhered not so much by their posterior extremities, but by their sides, thus forming irregular groups of 6–10 or 20 cells. As with *Tr. Brucei*, these agglutinated cells have their whips on the outside (Fig. 4, Plate 5). Frequently they attach themselves to red blood cells.

In addition to the agglutination groups, real multiplication rosettes are to be seen, though rather few in number. These, as in the case of *Tr. Lewisi* and *Tr. avium*, have their flagella on the inside (See Figs. 2 and 3, Plate 5). The older rosettes present

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a very characteristic appearance. The spindle-shaped or pyriform cells are on the periphery, while the central portion, 10 to 15 μ in diameter, shows a tangle of slowly waving flagella.

Further details regarding this species will be given under *Tr. Mesnili.*

Type 3.—This was found in a goldfinch, No. 353, in the stained blood of which only one trypanosome could be detected. As seen from Table II the measurements of this cell correspond closely with those of the small form of *Tr. avium*, so much so that if this were the only criterion there would be no hesitation in regarding it as of that species. However, the coarse granulation of the contents, as seen in Fig. 1, Plate 6, suggests a difference, and this is clearly substantiated by the results of cultivation. In view of the fact that this type is so clearly distinct from either *Tr. avium* or *Tr. Mesnili*, we must look upon it as a new species, and have named it, as already mentioned, *Tr. Laverani*, in honor of Dr. Laveran of the Pasteur Institute.

This trypanosome is characterized by a very slow growth in cultures. In such the flagellates are scarcely to be seen before the fifth day; they are moderately abundant about the tenth or twelfth day, and cannot be said to be rich until about the second or third week. Thus, the blood of the bird was planted on two tubes of the medium. Ten days later, each tube showed several rosettes and some actively motile trypanosomes. The maximum of growth was reached shortly before the twenty-third day, for at that time the growth was quite rich, but the motion had disappeared or nearly so. An examination on the thirty-fifth day showed masses of round bodies, many of which were also single. Tangles of free flagella were also common.

The slow growth in cultures, of *Tr. Laverani* distinguishes it at once from either of the preceding types. There are, however, other equally striking differences.

The rosettes are less common than in Type 2 and are perhaps smaller. They are composed of rounded, pyriform, or spindle-shaped cells which contain numerous bright yellowish-green globules, which are larger, and perhaps more abundant, than those of the preceding species. The cells taken as a whole are narrower

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than those of *Tr. Mesnili.* Their flagella, directed centrally, are more delicate and hence more difficult to see in the living preparation than are those of the preceding type (Fig. 3, Plate 7).

The free forms, while common enough, are much less motile than is the case with either of the preceding. It may often happen that in a preparation not more than a dozen cells can be found to travel about actively. When moving about they roll on their long axis, whip forward. Usually they attach themselves by their whips to the glass and show a slow swaying motion.

The absence of marked motion is apparently due to a stickiness of the exterior of the free cell. This is seen in the fact that a red blood corpuscle often adheres to the middle portion of the cell when touched by the latter. It is further indicated by the marked tendency to form agglutination groups, which are somewhat suggestive of those of *Tr. Brucei.* The two cells may adhere laterally, with the whips at opposite ends, and the group thus started may increase in numbers so that eventually several hundred cells may be together. In such groups the whips are on the outside and in active motion. The cells usually contain from 5–10 large globules which are found in the posterior end though not always.

Rounded, rolling forms about the size of a red blood corpuscle (Fig. 6, Plate 7) are common in the early cultures and represen dividing forms. In old cultures, the rounding up, as with other trypanosomes, is a common involution change.

The stained preparations show a coarsely granular nucleus, and close to it or anterior is a small blepharoplast. A most interesting feature is the presence of a terminal rod which we have not observed thus far with certainty in any other type. This rod usually lies right up against the wall at the posterior tip of the cell, and for that reason can be easily overlooked. In such a case, all that can be observed is a slightly deeper stain of the edge of the cell at that point. When, however, cell-division is taking place and especially if the preparation is thoroughly flattened out in the act of spreading, this body can be readily seen (Plate 6). That this terminal rod is a distinct structure cannot be doubted. It divides about the same time or a little later than does the

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nucleus, but does not approach the latter except possibly in the rounded forms. It is stained a lighter pink by the Romanowsky method than is the nucleus. Thus far we have been unable to make out any connection with the other structures of the cell. It is possible that a similar body is present at the anterior end, as suggested by some of the photographs.

In a recent paper on the development of Herpetomonas, Prowazek[75] describes a flat, spiral thread which passes from the blepharoplast to the posterior end of the body where it terminates in an "undeutlichen Doppelkorn." In the process of reduction previous to copulation this diplosome was said to divide, giving rise to a group of four granules, two of which divided again, so that a group of six granules resulted. The further changes of these bodies could not be followed.

As stated above we have looked closely for some connection with the other structures of the cell, but have not been able to observe any. Possibly, by giving special attention to the method of staining, something may be ascertained on this point. In the dividing cell two such rods are found, but whether the division occurs transversely or longitudinally cannot be said. This terminal rod can be made out more or less easily in the majority of the cells. There are some, however, in which for some reason this cannot be done.

The staining of the cultures of this type is much more difficult than of either of the preceding. The globules which are seen in the living cell do not readily stain, and hence may appear as colorless bodies or vacuoles. On allowing the stain to act for a longer time the globules may be stained a dark red, but in that case the nucleus, blepharoplast, and terminal rod cannot be made out in the deeply stained contents.

Sub-type 3a.—This was obtained from a brown thrasher, No. 386, and is designated as strain T. It is certainly closely related to, if not identical with, *Tr. Laverani.* Like the latter it is a slow grower, requiring from 12–14 days to yield a fair culture. Rosettes are common and like the preceding are made up of round bodies or spindles containing many large globules. Free round bodies 8–10μ in diameter are to be seen rolling about in

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early cultures. The free forms 15–18 μ long, unlike those of the preceding, have a narrow posterior end, are narrower and more tapering, and possibly more coarsely granular; they have a smooth gliding motion somewhat circular in course. They apparently are also quite sticky, since they tend to stick to the glass or to agglutinate in groups of 10–20 or more cells. The agglutination is perhaps not as marked as in the case of *Tr. Laverani.*

The length of the body of the free form is on an average 15–18 μ; at times cells 25 μ long are met with. The width varies from 2.5–4 μ. The free whip is from 5–10 μ long, but in the case of the round form it may be much longer. The blepharoplast is very small, usually not over 0.3 μ, and is anterior to the nucleus. The posterior end tapers to a point, whereas the anterior end is commonly blunt. The contents, especially in the anterior half, are rich in granules which stain deeply.

The round forms are, as a rule, about 6 μ in diameter. The smaller ones on division may give rise to cells that measure but $3 \times 5\,\mu$. These usually have a relatively long whip, 10–15 μ long. There is a faint suggestion of a terminal body, as in the case of *Tr. Laverani*, but further staining will be necessary to positively demonstrate this structure. The nucleus is apparently more difficult to stain than is that of the other types.

Type 4.—This was met with three times, and in cultures only. It was obtained twice from blue jays (Nos. 273 and 429), strains M and C′; and once from a rusty blackbird (No. 420) strain A′. In these cases the blood preparations, even on re-examination, failed to show the native trypanosome.

The cultural characteristics of the three strains were practically identical, and for that reason they may be taken to represent a single species. On the other hand, they present marked differences from the preceding types, sufficiently so, to entitle them to be considered as representing a distinct species.

The growth is very rapid, and as a result it is quite rich on the fourth or fifth, and reaches its maximum on about the eighth day. It shows the usual two forms—rosettes and wide, free-swimming cells.

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The rosettes are by no means as marked as with the other types. They are scarce and small, rarely consisting of more than about 20 cells. They may be composed of round bodies or of elongated spindles. The whips are on the inside. The spindle forms sway quite freely, sufficiently so as to detach themselves, and it is probably due to this that the rosettes are so few and small. The round bodies and spindles show only a fine granulation of the contents; and are thus in marked contrast to the two preceding types. The round bodies are not much larger than a red blood cell.

The free forms are numerous and move about very rapidly, whip forward. When in motion the cell turns on its long axis, and as a result it may have a wave-like appearance or lateral sway. The body is usually about 15 μ long, and is widest near the anterior end. The width is 3 μ or less. The posterior half may contain numerous small granules, unlike the large globules of *Tr. Mesnili* and *Tr. Laverani*. The undulating membrane can be readily seen at the anterior end.

Agglutination occurs as with the former two types by several cells sticking together lengthwise, the whips remaining on the outside. Large masses of cells may thus form. That this is largely due to a stickiness of the wall is shown by the readiness with which they attach themselves to red blood corpuscles. At times a single cell may be seen to travel about with two such corpuscles.

Sub-type 4a.—This is represented by strain X from goldfinch No. 354. It resembles the preceding but shows considerable variation. The rosettes are even scarcer and are rarely composed of more than a dozen cells which sway actively.

The free form is about 20 μ long and 3 μ wide. Smaller forms of 14–16 μ and larger ones of 24–26 μ are also present. The free whip is from 14–22 μ long. The relatively large blepharoplast (1–1.3 μ) is almost always on the side of the nucleus which is about 2 μ wide. This position of the blepharoplast and its large size distinguishes the cell at once for *Tr. Laverani*. The posterior part of the body is blunt, while the anterior part elongates along the whip to a fine point.

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DESCRIPTION OF SPECIES.

Before taking up the description of the species met with by us it will be well to consider briefly the several forms found by other observers and endeavor to correlate their findings as far as possible with our own. This is obviously a matter of some difficulty, owing to the entire absence of cultural characteristics.

The earliest observations, those of Danilewsky, indicated the presence of two forms of trypanosomes in the blood of birds, the small and the large form. As shown heretofore, Danilewsky was undoubtedly correct in the general assumption that these represented but one species, his *Trypanosoma avium*. The close agreement in form and size of his organisms with the majority of the trypanosomes studied by us leads us to accept them as identical, and for that reason we have designated our common species by the old name.

The description of *Tr. avium* as given by Laveran and applied to the organism found in an owl can, in the main, be reconciled with the above. The length, including the whip, is given as 33–45 μ. This, it will be seen, corresponds to the smaller forms mentioned, which measure, without the whip, 20–25 μ. The position of the centrosome, near the posterior end, accords better for the small form than for the large one.

The form which Hanna met with in the blood of pigeons, in India, certainly resembles very closely the large type, such as is shown in Plate 3, and may therefore be looked upon as likewise falling under *Tr. avium*. The same may be said of the trypanosome which he describes as present in the Indian crow, but which Dutton and Todd apparently refer to as in a blue jay.

The short, broad trypanosome which Dutton and Todd found in Senegambia is in form, size, and position of the centrosome the same as the small forms found by us, as will be seen on comparing our photographs with their illustration. It can therefore be regarded as *Tr. avium*.

The *Tr. Johnstoni* of Dutton and Todd certainly represents a distinct species. We have found one single specimen, in a stained preparation of the blood of the woodpecker, No. 391, which resembled somewhat this species. Unfortunately the slide

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was misplaced, and as no cultures were made we are unable to give any detail.

The most recent species of bird trypanosome is the *Tr. paddæ*, described by Laveran and Mesnil, and by Thiroux. Were it not for the fact that this organism is said to have a very short free whip, it might well be taken to correspond with the large S-form which is finely striated, as shown in Plate 3. It is to be hoped that cultures of this organism will be made, in which case it will be possible to make an exact comparison with those of ours.*

TRYPANOSOMA AVIUM, DAN., N. & MACN. EMEND.

This is apparently the most common species found in birds, having been met with twenty-five times in the blackbird, blue-bird, blue jay, oriole, robin, English sparrow, and song sparrow. This does not include the sub-type 1*a*, previously mentioned, which was found four times, namely, in the mourning dove, blue jay, and flicker.

The native trypanosome may appear in either the small or the large form, or in both. These two forms have already been described, and for that reason, in order to avoid repetition, the reader is referred to that portion of the paper.

It grows very readily on the blood agar medium, and, as a rule, rich cultures are obtained on the fourth to the sixth day. The culture is characterized by the presence of two strikingly different forms, the multiplication rosettes and the free-swimming, darting, thread-like spirochetes.

The rosettes are made up of smaller cells and are a more prominent feature of the culture of *Tr. avium* than are the like formations in the case of the other species. The long, slender spirochete form is apparently distinctive, since the corresponding free-swimming forms of the other species are shorter and wider. Moreover, the blepharoplast in the latter is by the side of or

* As this paper is going to press we learn from Dr. Thiroux that he has succeeded in cultivating this trypanosome which is now in its third generation. An examination of some preparations made from these cultures and kindly sent to us by him show at a glance that this organism is entirely distinct from our Tr. avium. The cultural forms more nearly approach those of Tr. Laverani. Dr. Thiroux has also shown that the injection of Tr. paddae into canaries and paddas is not followed by a halteridium infection and hence that these two organisms are entirely distinct, thus confirming our results and the view expressed at head of page 273.

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anterior to the nucleus, whereas in the spirochete it is considerably posterior to this structure.

An extended description of these two forms is given on pp. 282 and 283.

The attempts at infection of birds with cultures of this trypanosome were far from satisfactory. It should be stated, however, that most of these inoculations were made with the object of finding intracellular parasites, which according to Schaudinn's views should be expected, and consequently the search for the trypanosomes was not as thorough as it might have been. The culture fluid used for the injections was either derived from a single strain, or in some instances a mixture of several was used. The injections were as a rule intrapleural, and the fluid was always extremely rich in trypanosomes. The usual dose was from 0.1–0.2 c.c. One screech owl, two pigeons (squabs), three chickens (ten days old), seven robins, twenty-two sparrows, and six old canaries were used. The work with robins and sparrows was rather unsatisfactory on account of the difficulty of keeping the birds alive for any length of time. In only one of these birds, a canary, was a positive result obtained.

This canary received an injection of a very rich culture of strain D. Four days later, it showed a marked leucocytosis, and three trypanosomes were found in the fresh blood. These were in the form of a long, straight spindle, about 4.5 μ wide and 28 μ long, with a free whip measuring 12–15 μ. The undulating membrane was very conspicuous, starting from near the posterior end and passing over the body in two large waves. The nucleus was readily visible as a large oval, about as wide as the parasite. Fine granules were present, especially in the posterior part. The motion was rather sluggish and confined to the neighborhood of the one field of the microscope. The next two examinations, made about a week apart, were negative. On the third and fourth week a single trypanosome was found each time. It then appeared to be smaller than on the previous occasion. The body was about 17 μ long, three to four μ wide, and the whip measured about eight μ. Three examinations in the course of the next two weeks were again negative. The trypanosomes were thus

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detected, off and on, for eleven weeks, and were found in the blood at the time of death. Although many attempts were made to obtain stained preparations of the trypanosome, they all failed, owing to the extreme scarcity of the parasite. Immediately after death cultures were made from the heart-blood of this canary, and in four days they showed a rich growth, consisting of the typical rosettes and spirochetes of *Tr. avium.* This culture, known as strain Y, is not included in Table I, since it was derived from an artificially infected bird. It makes the thirtieth strain isolated from birds.

TRYPANOSOMA MESNILI, N. SP.

This trypanosome was found in the blood of a red-shouldered hawk (*Buteo lineatus*), associated with *halteridium* and *H. Ziemanni.* The organism was very scarce, since only one specimen could be found on four slides, each of which was systematically searched by means of a movable stage.

The native trypanosome (see Fig. 1, Plate 5) is characterized by its large size and great bulk, and by a wide, rounded posterior extremity. The length of the body is 50 μ, and the width opposite the nucleus is 8 μ. The width of the cell at the posterior end is 5 μ. The distance from the posterior end to the centrosome is 7 μ, and from this to the nucleus it is 10 μ. The anterior portion of the body, which is bordered by a thick, undulating membrane, tapers rather abruptly to a narrow end which undoubtedly terminates in a free flagellum. This, however, cannot be made out in the specimen. The trypanosome was not seen alive in the blood.

The contents of the cell stain heavily and are coarsely granular. The centrosome is round and about 1 μ wide. The nucleus is relatively small and is found near the side having the undulating membrane.

The cultures of this organism grow very rapidly, and are very rich on the sixth to the seventh day. After that they undergo involution changes, forming large masses of very granular, round bodies. A few motile cells may be met with in such cultures, even at the end of six weeks.

The cultures show two types of cells, resembling in a general

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way those met with in *Tr. Lewisi* and *Tr. avium*. The one type makes up the multiplication rosette, while the other is free and swarming, and corresponds to the spirochete stage of *Tr. avium*.

The multiplication rosettes (see Figs. 2 and 3, Plate 5) vary considerably in size. The smaller ones are about 10 μ in diameter, and are made up of cells in process of active division. Such cells are about 3 μ wide and 5–6 μ long, and on division yield cells which are about 2 μ wide and 4 μ long. On account of the rapid division, the free flagella are very short and can scarcely be made out.

The larger rosettes consist of more fully developed cells, 10–100 in number. These are usually about 10 μ in length, but they may attain a width of 6 or even 7.5 μ. The full-sized spindles measure 4×15 μ. The free whips, about as long as the cell, are easily seen in the central portion of the rosette. On account of the length of the whips the cells composing such rosettes exhibit a marked swaying motion. The large oval or pyriform cells stain deeply, and are filled with dark granules and bright refracting globules. When free, as is often the case, they may be seen rolling about in the fluid.

The free, swarming cells, as stated above, correspond to the spirochete stage of *Tr. avium*. Unlike the spirochetes of the latter, they are short and very wide, and are provided with very long, free flagella. Such free cells ordinarily measure 4–6 μ in width and 20–25 μ in length, not counting the whip, which often measures 17–20 μ. From the widest portion, near the anterior end, the body gradually tapers toward the posterior extremity, which is either blunt or slightly pointed. On account of their large size, the undulating membrane can be seen at the anterior end, and for the same reason these cells have a stately, gliding motion. They travel rapidly, whip foremost, either in a straight line or in a circle, and often may be seen to roll on their long axis.

When the growth is very rapid, as a result of a specially favorable medium, the cells show only very minute globules, scattered all through the plasma. At other times the granules are

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rather large, and have a distinct yellowish green color. They are most abundant in the posterior half of the cell.

We have pointed out in connection with our other work on the cultivation of trypanosomes that the presence of bacteria tends to destroy these protozoa. The result is largely dependent on the kind of bacteria which chance to be present. In one instance, Laveran and Mesnil found the culture of *Tr. Lewisi* to remain alive and virulent in the presence of bacteria for about two weeks. A similar result, extending over a much longer period of time, we have noted in connection with *Tr. Mesnili.* A culture of this organism developed a contamination with a slow-growing streptococcus, and inasmuch as the trypanosome continued to grow, even luxuriantly, it was transplanted and subcultures were kept up. These have now been maintained for over three months, and, if anything, the mixed cultures are somewhat better than the pure ones.

The free, swarming trypanosomes show a considerable tendency to agglutination. This usually begins by two cells overlapping at their posterior ends. Other cells attach themselves to these by their sides, and thus large masses soon form. At times this agglutination takes place so rapidly under the cover-glass that, in the course of an hour or two, nearly all of the cells gather into these irregular clumps. In such agglutination groups the flagella are always on the outside. The undulating membranes and whips continue their motion, and as a result the entire mass can be seen to travel through the field.

The fact that the cells agglutinate along their whole length would seem to indicate that the surface of the body is more or less sticky. This view is substantiated by the behavior of these cells with respect to blood corpuscles. In some cultures it is a common occurrence to find the trypanosomes moving rapidly about with a red blood cell attached to the median portion. This could hardly happen unless the outside of the trypanosome was somewhat gelatinous. There is also a tendency, especially for the larger forms, to attach themselves by means of their whip to the glass surface.

The free motile forms can be seen in all stages of division.

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For this purpose the cell first shortens to a large pyriform body about 10 μ wide and 15 μ long. The whip likewise shortens. The blepharoplast divides first, then the nucleus, and finally the body itself begins to split (Fig. 5, Plate 5).

Only two injections of cultures of this trypanosome were made, and both were negative. A young screech owl (*Megascops asio*) and three young chickens (10 days old) were used.

TRYPANOSOMA LAVERANI, N. SP.

This interesting species was met with but once and that in goldfinch, No. 353. It was overlooked in the examination of the fresh blood and in the stained preparations. When, however, the culture was obtained from the heart-blood the original stains of the blood were re-examined by the help of a movable stage, with the result that one trypanosome was found on three slide smears.

The native trypanosome is shown in Fig. 1, Plate 6. It is a wide spindle which measures 20 μ in length and 6 μ in width. The blepharoplast is close to the sharp-pointed posterior end, about 1 μ distant. The photograph shows no free whip, but this must be considered as due to defective staining since in the cultures a long free flagellum is easily observed. The posterior part of the cell is coarsely granular which peculiarity is brought out very clearly in the cultures. The wide nucleus occupies the middle of the cell.

In artificial culture the trypanosome is characterized by a very slow and sparse growth.

The multiplication rosettes are rather few in number and are small. They consist of rounded or pyriform bodies, which eventually elongate to oval or spindle-shaped cells. In the living preparation these are found to contain numerous granules and bright yellowish-green highly refracting globules which do not readily take the dye, and consequently in stained preparations appear as colorless globules (see Plate 7). The globules are usually about 1 μ in diameter, but at times they may be found twice that size.

The spherical forms which may be looked upon as giving rise to the rosette are frequently found free and in active division.

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The blepharoplast divides first, then the nucleus, and lastly the protoplasm. The division is longitudinal and uneven, that is to say, the young cell may be only half as wide as the remaining part of the parent cell. The latter may therefore pass on to a second or third division, and, as a result, groups of three or four of such cells are often found. Such round or oval bodies usually measure $8 \times 10\mu$, but may attain a size of $12 \times 14\mu$. A noticeable feature in these bodies is the terminal rod which usually lies against the posterior wall, but may at times approach the nucleus. The free whips on these forms are relatively short, about $10–14\mu$.

The free forms appear, either as slender spindles, which have a fairly constant width along the entire length of the body, or as spindles which are appreciably wider in the middle portion and taper toward both ends. The latter are from $14–20\mu$ in length and $4–5\mu$ in width, and have a free whip which is as long or even longer than the body. The posterior end may be sharp but more often is blunt or cut off square, and shows the peculiar terminal rod. The slender spindle, mentioned above, measures from $20–25\mu$ in length and $2.5–3\mu$ in width. The whip is relatively shorter than in the preceding type, measuring about $10–14\mu$. In both forms the blepharoplast which is comparatively small lies anterior to the nucleus. It is usually about 0.7μ long, but in the dividing forms it may attain a length of nearly 2μ.

The free form divides by longitudinal fission without shortening or rounding up to any appreciable extent. The blepharoplast divides first, and this leads to the formation of a new whip and then, very often, to a division of the anterior portion of the cell. The nucleus then divides and the cleavage continues until complete division results. The terminal rod apparently divides after the nucleus, and, at the stage of complete division, two of these bodies can be seen at the posterior end.

The relatively feeble motion and the marked tendency to agglutinate have been mentioned under Type 3.

No inoculation experiments have as yet been made with this culture.

UNNAMED SPECIES.

The three strains described under Type 4 without doubt represent a distinct species, but inasmuch as the native trypanosomes

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have not been found, even after repeated search of the stained preparations of the blood, it has not seemed desirable at present to give this type a definite name. Inoculation experiments are now in progress and may help to supply this deficiency.

As to the sub-types 1*a*, 3*a*, and 4*a*, the opinion may also be expressed that they belong to new species. It has not, however, been possible up to the present time to give these a very thorough study, especially of the stained preparations of the cultures, and consequently they must be reserved for another occasion.

RELATION OF TRYPANOSOMES TO THE CYTOZOA.

This subject has acquired especial importance in view of the work of Schaudinn on the halteridium and the "leucocytozoön" of Danilewsky. The main conclusions arrived at by this investigator have been given in the introduction, and as already indicated they are not substantiated by our work.

In the first place we have shown that trypanosomatic infection of birds is widespread. Moreover, there is sufficient evidence of the existence of a large number of species of trypanosomes, and, as can be seen in Table I, a given species of bird (bluejay, goldfinch) may be subject to infection by two or three kinds of trypanosomes.

Double, triple, or even quadruple infection in birds is not an uncommon occurrence, and on that account it is not surprising to find trypanosomes associated with one or more intracellular parasites. Such association, however, cannot be said to have any other significance than that of mere chance or accident. The two extremes: first, of cytozoa without trypanosomes; and second, trypanosomes without cytozoa, are common enough as has been shown. Furthermore, it has been made evident that a given cytozoön, as, for example halteridium, may be associated with at least three distinct species, *Tr. avium*, *Tr. Mesnili*, and *Tr. Laverani*. Again, an examination of Table I will show in the case of the robin that the same species, *Tr. avium*, may be associated with any one of four cytozoa. The remarkable uniformity of the occurrence of this species of trypanosome under these conditions is difficult of explanation from the stand-

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point taken by Schaudinn. It is also worth noting that in the one case of infection with the "leucocytozoön" *H. Ziemanni* we have obtained a trypanosome, *Tr. Mesnili*, which in cultural characteristics is totally different from the *Spirochete Ziemanni* described by Schaudinn as a stage of this cytozoön.

In view of the ease with which trypanosomes can be cultivated in the test-tube it is reasonable to suppose that the same result can be obtained with the mosquito. That is to say, the few trypanosomes which may chance to be present in the blood sucked up by the mosquito rapidly multiply in the stomach of the insect and give rise to rich cultures similar in every respect to those met with in the test-tube. Thus, the multiplication rosette, with the whips directed centrally, will be found as the predominating feature with a given species of trypanosome. In the case of *Tr. avium* the long, slender, free forms or spirochetes are especially noticeable.

It seems to us, therefore, that the observations of Schaudinn are open to an entirely different interpretation than that given by him. It appears that he has cultivated trypanosomes *in vivo* and has obtained forms which agree fully with those obtained by us in artificial culture, *in vitro*.

Assuming the identity of the flagellates found by Schaudinn and ourselves *in pure culture*, it can be expected that like results would be obtained by the injections, on the one hand, of suspension of the mosquito culture, and on the other hand of the culture fluid from the test-tube. On this point we have made a very large number of inoculations into sparrows, robins, owls, canaries, young pigeons, ring doves and very young chickens, but have never been able to obtain any indication of the development of intracellular parasites as a result. The amount of culture fluid thus injected into a bird was incomparably greater than could be obtained by the injection of a large number of mosquitoes. It is true that with one exception we failed at the same time to infect the birds with trypanosomes.

Since the injection of suspensions of the infected mosquitoes, or the mere bite of a few of these insects, as shown by Schaudinn and by the Sergents, is capable of causing an infection with the

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intracellular parasites, such as the halteridium, it follows that one of two conclusions can be drawn. First, it may be held that the flagellates observed by Schaudinn in the mosquito are the real stages of the cytozoön and that they are wholly distinct from the trypanosomes found in birds. Or, second, it may be held that the flagellates found in the mosquito are derived from the trypanosomes in the birds, and as such have nothing to do with the intracellular parasites; in which case the positive infection with the mosquito must be due to some as yet unrecognized stage of the cytozoön. This latter view is, in our opinion, the correct one.

In order to furnish a definite solution of this question it will be necessary to allow mosquitoes to bite birds which by the cultural method have been shown to be free from trypanosomes. If flagellates appear in the stomach of such mosquitoes, after feeding on birds known to be free from trypanosomes, it will be justifiable perhaps to consider them as stages in the development of cytozoa. On the other hand, since only about 10 per cent of the mosquitoes which feed upon the infected bird develop flagellates (according to the Sergents about 25 per cent), it ought to be possible to produce with those which show no trypanosomes the cytozoön infection.

The recent detection by Rogers[76] of trypanosomes in so-called culture, made with the blood of patients infected with *Piroplasma Donovani* is assumed by some to indicate that the trypanosome is a stage in the development of that parasite. This position, however, is far from being established. It may well be asked, in view of the great frequency of trypanosomatic infection of birds, whether or not a similar condition may not obtain with man, especially in the tropics. The direct detection of such trypanosomes in the blood would be perhaps as difficult of accomplishment as it is in the case of birds, and for that reason they could be easily overlooked. It is probable that if systematic cultivations are made of the blood of a large number of individuals inhabiting the warm countries, conditions will be found to parallel the infection of birds, rats, fish, and amphibians. On *a priori* grounds one could expect to find relatively harmless trypanosomes in the blood of man besides the one pathogenic *Tr. gam-*

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biense. The recovery from trypanosomatic infection of the one known case, that of a European, may be looked upon as due to the presence of a different trypanosome from that of sleeping sickness. Similarly, the observation of Rogers may be interpreted as indicating the existence of a hitherto unrecognized form of trypanosomatic infection in man.

SUMMARY.

The main results arrived at in this investigation can be briefly summarized as follows:

1. Trypanosomatic infection of birds is very common and widespread.

2. The different species of birds may harbor different species of trypanosomes; and the same species of bird may be infected by several species of these parasites.

3. The trypanosomes may or may not be associated with intracellular parasites; and no constancy can be shown to exist between a given trypanosome and a given cytozoön.

4. The *Trypanosoma noctuæ* and the *Spirochete Ziemanni* of Schaudinn probably represent trypanosomes which have multiplied in the mosquito; and are not to be considered as stages in the life-history of cytozoa.

5. The unsuspected frequency of trypanosomes in birds makes it probable that, in the tropics, animals and even man may be found to harbor in small numbers such parasites. The presence of trypanosomes in the blood of man does not necessarily mean an infection with *Tr. gambiense.*

In conclusion it is a pleasure to acknowledge the aid given by the Rockefeller Institute in the furtherance of this work. Our thanks are also due to Dr. J. F. Eastwood for his great help in the photographic room, and to Mr. H. N. Torrey, the present holder of the Rockefeller fellowship, for his invaluable assistance in the work of cultivation and staining.

EXPLANATION OF PLATES.

These illustrations were taken by means of the large Zeiss microphotographic apparatus. With the exceptions noted the objects are all magnified 1,500 diameters. They are all stained by the Romanowsky method, except Fig. 5, Plate XI, in which case a living preparation was used.

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PLATE 1.—NATIVE TRYPANOSOMES IN THE BLOOD OF BIRDS, *Tr. avium*.

FIG. 1.—Trypanosome giving rise to strain A, in mourning dove, No. 5.

FIG. 2.—The same of strain B, in mourning dove, No. 6.

FIG. 3.—The same of strain C, Baltimore oriole, No. 41.

FIG. 4.—Another slide of the same blood as preceding, showing the crushed form.

FIG. 5.—Trypanosome of which no culture was obtained, from Baltimore oriole, No. 272.

FIG. 6.—Another slide of the same blood as the preceding, showing a slightly crushed form. Note flagellum.

PLATE 2.—NATIVE TRYPANOSOMES IN THE BLOOD OF BIRDS, *Tr. avium*.

FIG. 1.—Trypanosome giving rise to strain G, robin, No. 50.

FIG. 2.—The same of strain D, robin, No. 51.

FIG. 3.—The same of strain F, robin, No. 54.

FIG. 4.—From same preparation as preceding, showing the trypanosome slightly flattened. To the left a cell infected with a female form of *Hæmoproteus majoris*, Lav. This shows in the center a faintly stained nucleus, while adjoining and overlapping it is the large deeply stained blepharoplast.

FIG. 5.—Small form of trypanosome, of which no culture was obtained, from robin, No. 270.

FIG. 6.—Large S-shaped trypanosome in the same preparation as the preceding. The undulating membrane is particularly well developed. The nuclear portion is not stained.

PLATE 3.—NATIVE TRYPANOSOMES IN THE BLOOD OF BIRDS, *Tr. avium*.

FIG. 1.—Small form of trypanosome giving rise to strain K, in blue jay, No. 244.

FIG. 2.—Another preparation of the same blood showing the crushed form.

FIG. 3.—Large trypanosome S-shaped in the same preparation as that of Fig. 1. The blepharoplast, surrounded by achromic zone, is near the nucleus. Note the striations on the surface of the cell.

FIG. 4.—Large form in same preparation as preceding.

FIG. 5.—Small form of trypanosome of strain R, in blue jay, No. 278. Note the free flagellum.

FIG. 6.—Large S-shaped trypanosome in same preparation as preceding.

PLATE 4.—NATIVE TRYPANOSOMES IN THE BLOOD OF BIRDS, *Tr. avium*.

FIG. 1.—Trypanosome giving rise to strain E, in robin, No. 53.

FIG. 2.—The same of strain H, in song sparrow, No. 142.

FIG. 3.—Small form of trypanosome, of which no culture was obtained, in song sparrow, No. 288.

FIG. 4.—Large form in same preparation as preceding.

FIG. 5.—Trypanosome, of which no culture was obtained, in blackbird, No. 401.

FIG. 6.—Another in same preparation as preceding.

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PLATE 1.

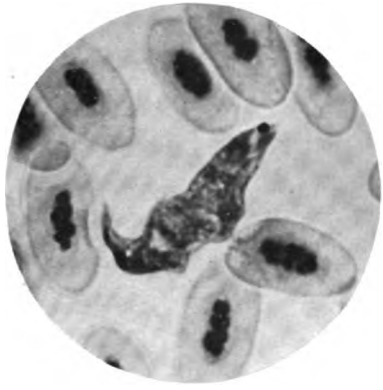

FIG. 1.

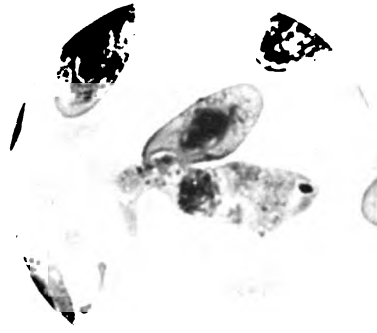

FIG. 2.

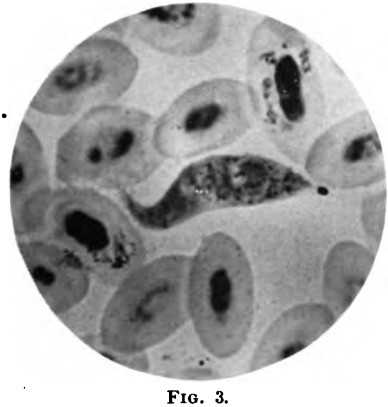

FIG. 3.

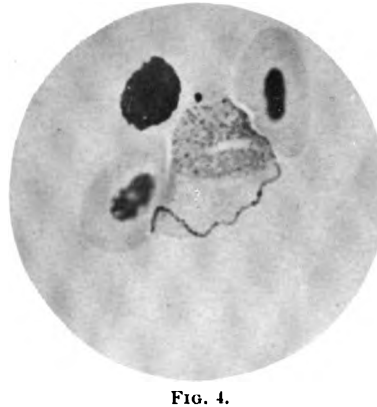

FIG. 4.

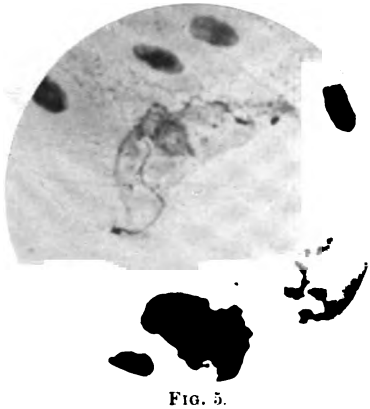

FIG. 5.

FIG. 6.

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PLATE 2.

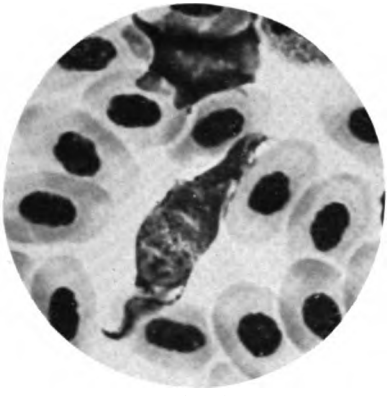

FIG. 1.

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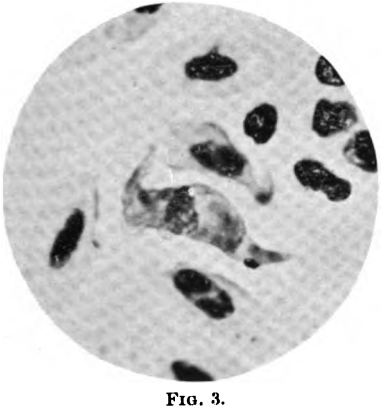

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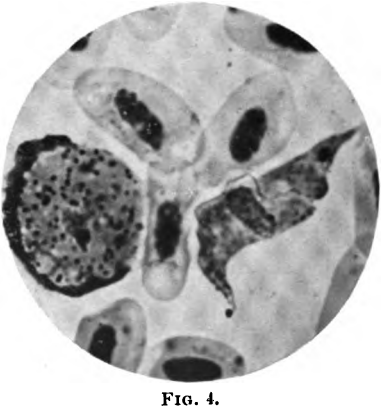

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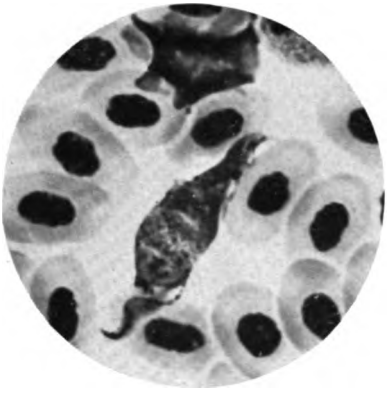
PLATE 2.



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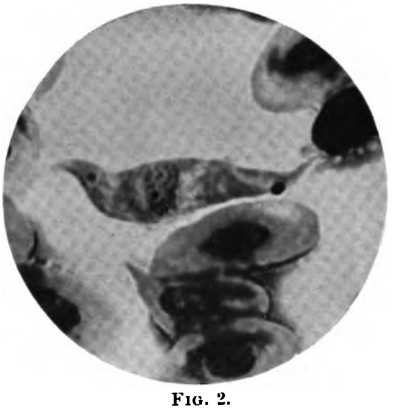

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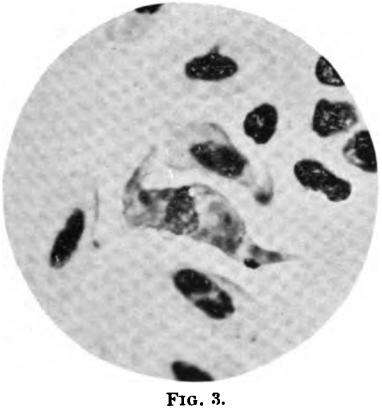

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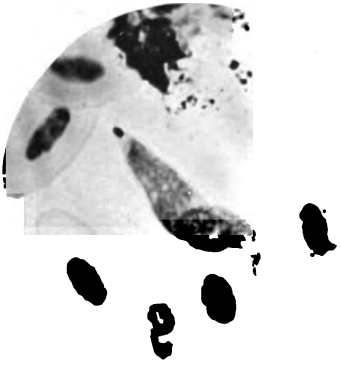

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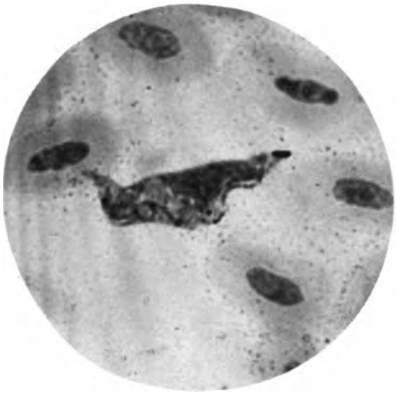
PLATE 3.



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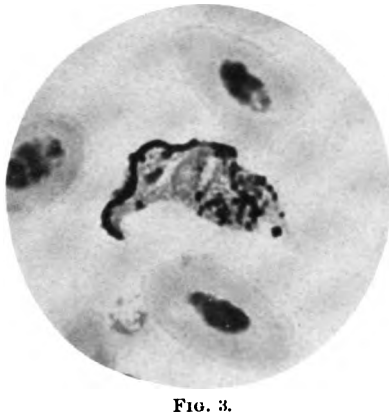

FIG. 3.

FIG. 3.

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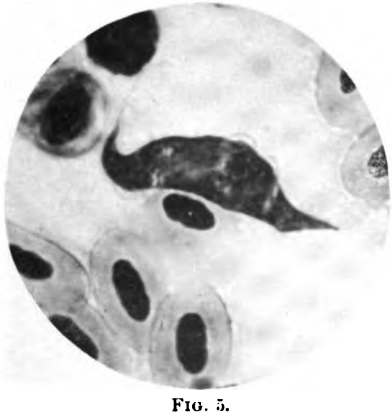

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PLATE 4.

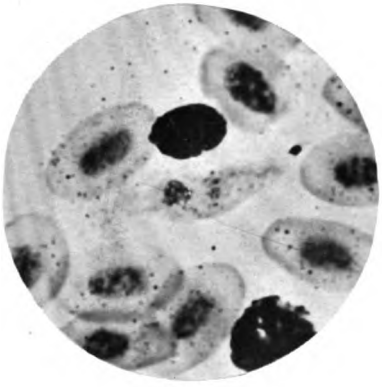
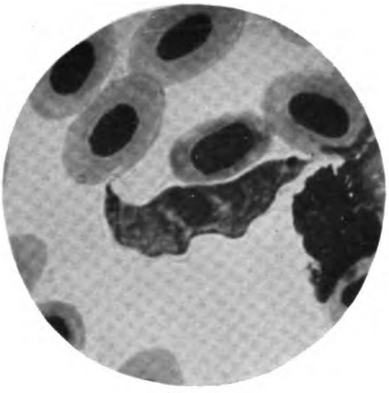


FIG. 1.



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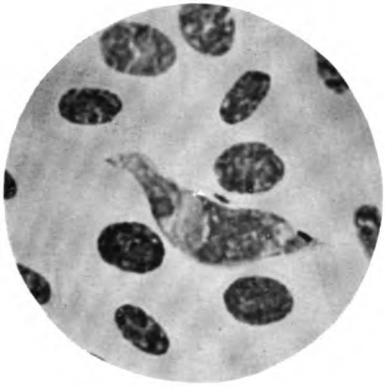

FIG. 3.

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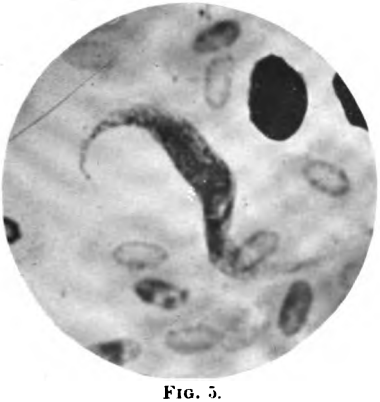

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PLATE 5.

FIG. 1.

FIG. 2.

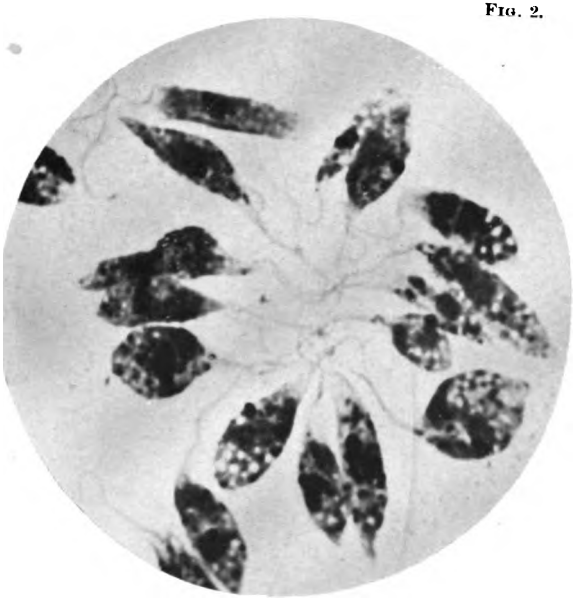

FIG. 3.

FIG. 4.

FIG. 5.

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intracellular parasites, such as the halteridium,
of two conclusions can be drawn. First, it may be
flagellates observed by Schaudinn in the mosquito are the
stages of the cytozoön and that they are wholly distinct from the
trypanosomes found in birds. Or, second, it may be held that
the flagellates found in the mosquito are derived from the try-
panosomes in the birds, and as such have nothing to do with the
intracellular parasites; in which case the positive infection with
the mosquito must be due to some as yet unrecognized stage of
the cytozoön. This latter view is, in our opinion, the correct one.
In order to furnish a definite solution of this question it will
be necessary to allow mosquitoes to bite birds which by the cul-
tural method have been shown to be free from trypanosomes. If
flagellates appear in the stomach of such mosquitoes, after feeding
on birds known to be free from trypanosomes, it will be justifiable
perhaps to consider them as stages in the development of cytozoa.
On the other hand, since only about 10 per cent of the mosquitoes
which feed upon the infected bird develop flagellates (according
to the Sergents about 25 per cent), it ought to be possible to
produce with those which show no trypanosomes the cytozoön
infection.

The recent detection by Rogers[78] of trypanosomes in so-called
culture, made with the blood of patients infected with *Piroplasma*
Donovani is assumed by some to indicate that the trypanosome is
a stage in the development of that parasite. This position, how-
ever, is far from being established. It may well be asked, in
view of the great frequency of trypanosomatic infection of birds
whether or not a similar condition may not obtain with man
especially in the tropics. The direct detection of such trypano
somes in the blood would be perhaps as difficult of accomplish
ment as it is in the case of birds, and for that reason they cou
be easily overlooked. It is probable that if systematic cultiv
tions are made of the blood of a large number of individu
inhabiting the warm countries, conditions will be found to para
the infection of birds, rats, fish, and amphibians. *On a pr*
grounds one could expect to find relatively harmless trypa
somes in the blood of man besides the one pathogenic *Tr. g*

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Fig. 2.

Fig. 3.

Fig. 4.

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PLATE 6.

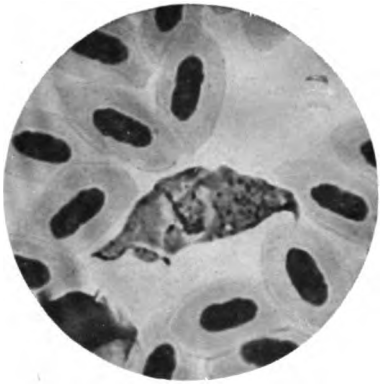

FIG. 1.

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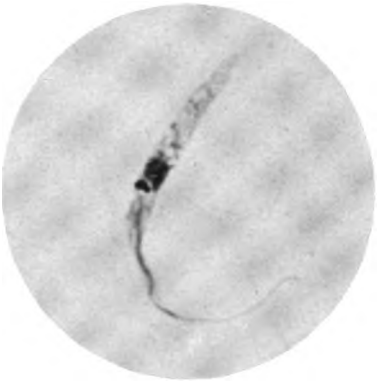

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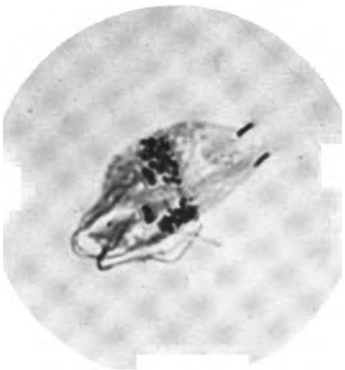

FIG. 6.

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PLATE 7.

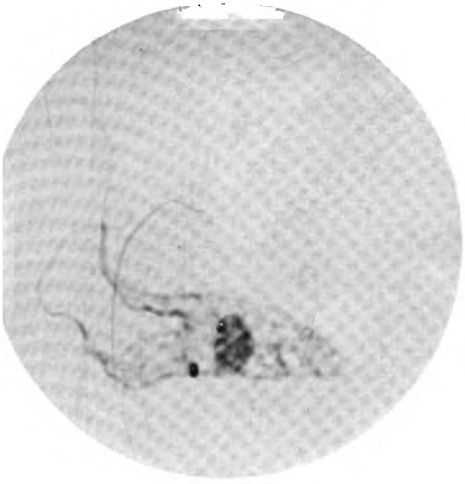
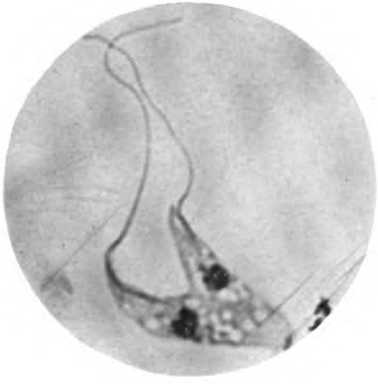


FIG. 1.



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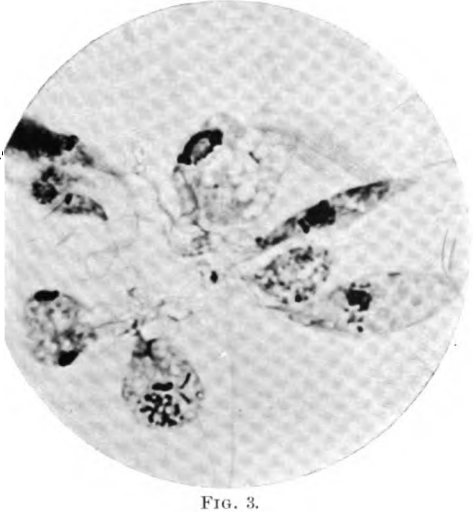

FIG. 3.

FIG. 4.

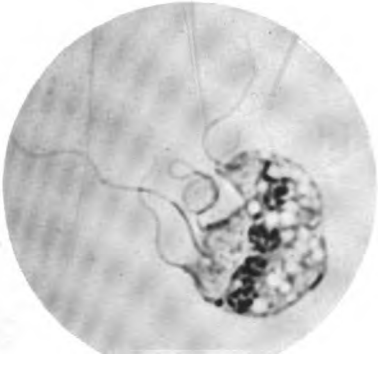

FIG. 5.

FIG. 6.

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PLATE 7.

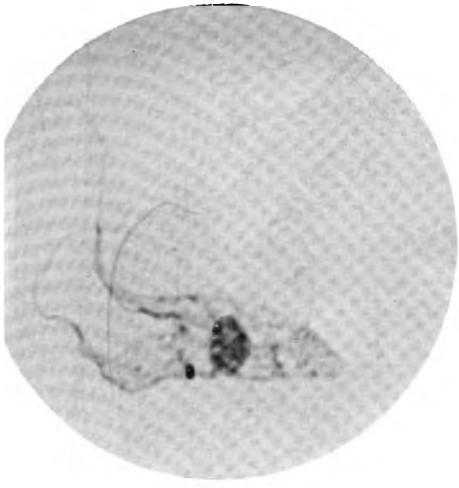
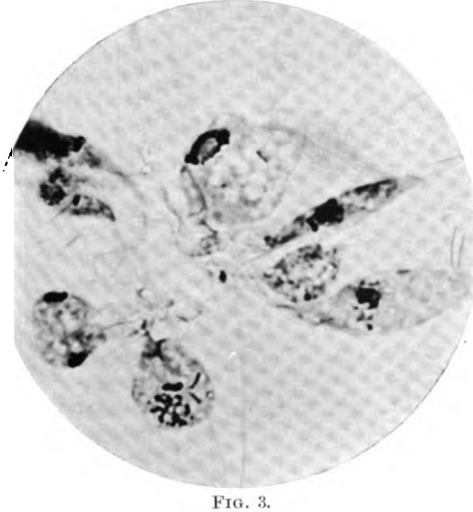


FIG. 1.

FIG. 2.



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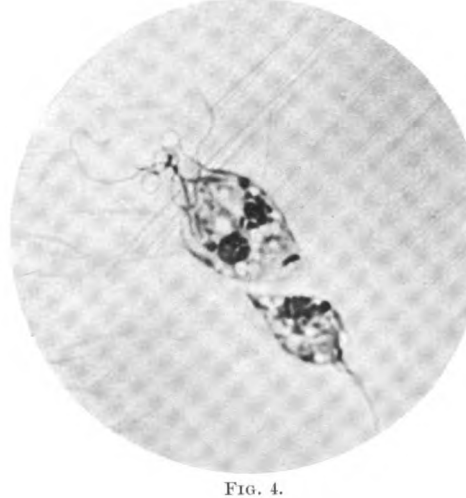

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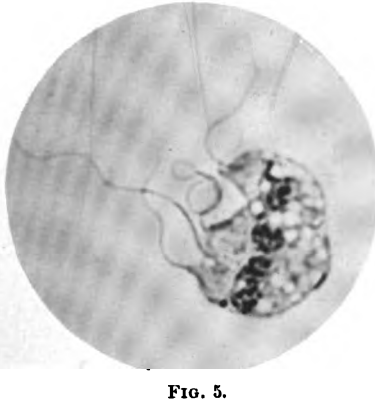

FIG. 5.

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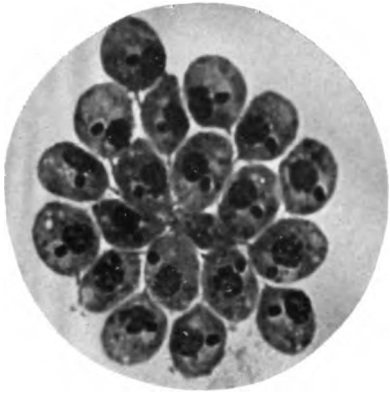
PLATE 8.



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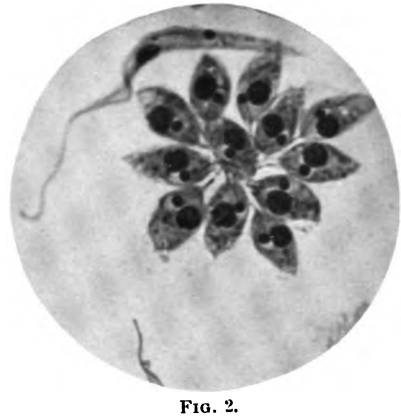

FIG. 2.

FIG. 4.

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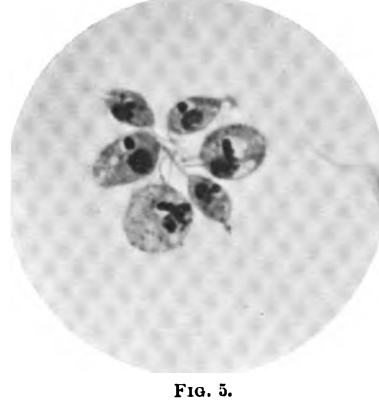

FIG. 5.

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PLATE 9.

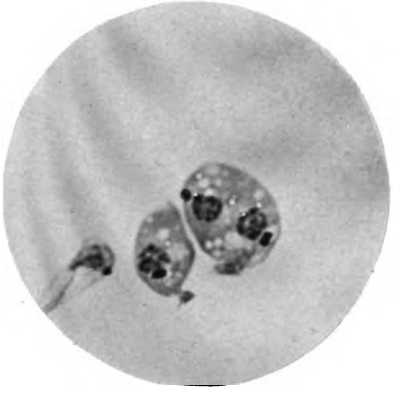

FIG. 1.

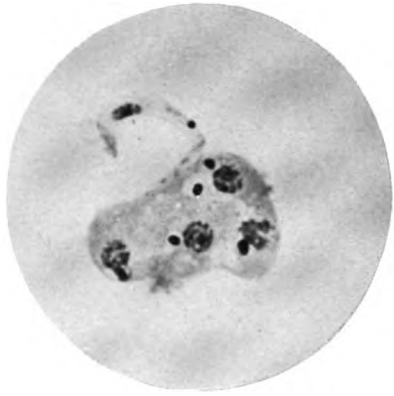

FIG. 2.

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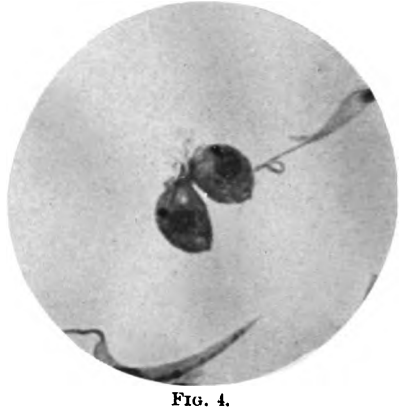

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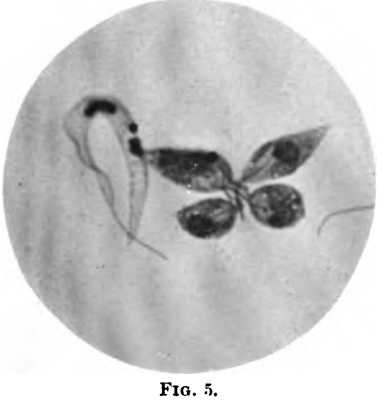

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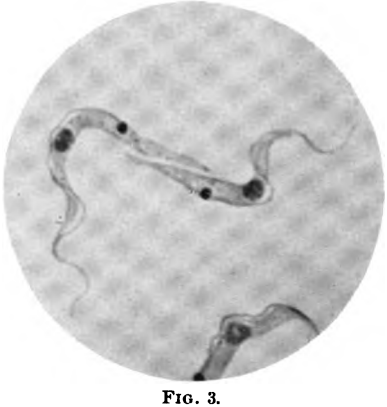
PLATE 10.

FIG. 1.



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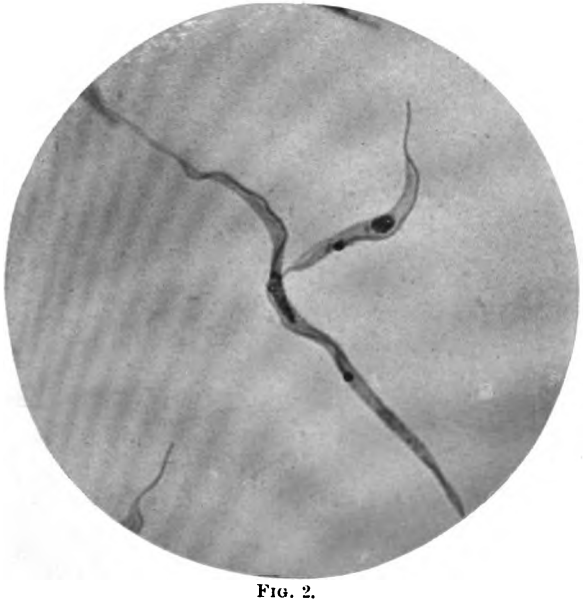

FIG. 2.

FIG. 4.

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PLATE 11.

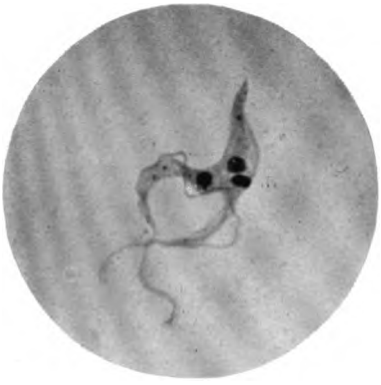

FIG. 1.

FIG. 2.

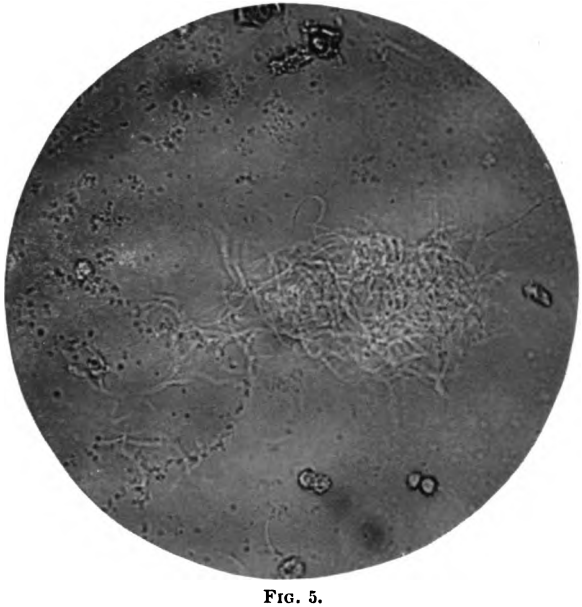

FIG. 5.

FIG. 3.

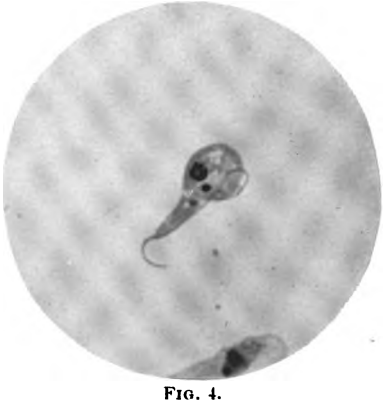

FIG. 4.

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PLATE 5.—NATIVE AND CULTURAL FORMS OF *Tr. Mesnili*, n. sp.

FIG. 1.—*Tr. Mesnili* in blood of hawk, No. 350, source of strain P.

FIG. 2.—Culture of *Tr. Mesnili* on blood-agar, gen. 6, five days at 25°. Small multiplication rosette consisting of large and small cells, some in process of division. The flagella are central and short.

FIG. 3.—From same preparation as preceding. Large multiplication rosette with full-grown cells, some dividing. The flagella are long and centrally directed.

FIG. 4.—From same preparation as preceding; agglutination group of five swarming cells. Reduced from 1500 × to about 1160 ×.

FIG. 5.—From same preparation as preceding. A free-swarming form, like that shown in Fig. 4, in process of division. In the center are two faintly stained nuclei while the two blepharoplasts are deeply stained.

PLATE 6.—NATIVE AND CULTURAL FORMS OF *Tr. Laverani*, n. sp.

Note in the latter the presence of the terminal rod at the posterior end; also the smallness of the blepharoplast and its position, anterior to the nucleus.

FIG. 1.—*Tr. Laverani* in blood of goldfinch, No. 353.

FIG. 2.—Free form with blunt end, culture 12 days at 25°, Gen. 6.

FIG. 3.—Free tapering form. From same slide as preceding.

FIG. 4.—The slender or narrow free form. This and the next two are from a culture in Gen. 7, grown six days at 25°.

FIG. 5.—The free form in process of division; note the two nuclei, two blepharoplasts, and two flagella; also the presence of colorless globules.

FIG. 6.—Dividing form, with two nuclei and two blepharoplasts; each of the latter is in process of division as shown by the fact that each gives off two flagella. Note the prominent terminal rods.

PLATE 7.—CULTURAL FORMS OF *Tr. Laverani*.

These were obtained from the seventh generation grown at 25° for 6 days. Note the prominent terminal rod and the position of the blepharoplast, anterior to the nucleus; also the presence of the colorless globules.

FIG. 1.—Dividing free form with one nucleus and two blepharoplasts.

FIG. 2.—The same as Fig. 1, but with two nuclei and two blepharoplasts. Compare this and the preceding figures with Fig. 5, Plate 5, and Fig. 1, Plate 11.

FIG. 3.—Multiplication rosette of pyriform and spindle-shaped cells. Note the tangle of flagella at the center.

FIG. 4.—Large and small oval bodies, the former in process of division. Note that each blepharoplast is giving off two whips, which indicates that another division is about to occur.

FIG. 5.—Like Fig. 4, but the division has gone on resulting in the formation of four nuclei, and four blepharoplasts, and four whips.

FIG. 6.—Earlier stage of the round form than is shown in Figs. 4 and 5. One nucleus, two blepharoplasts, and two whips.

PLATE 8.—CULTURAL FORMS OF *Tr. avium*.

Strain Q from robin, No. 257, gen. 7, grown seven days at 25°. The figures on plates 8, 9, and 10, and figs. 1, 2, 3, 4 of Plate 11 were made from the same slide.

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FIG. 1.—Early type of multiplication rosette consisting of round bodies. Shows whips on the inside.

FIG. 2.—Another multiplication rosette, cells oval in form. One spirochete in the field. Whips as before.

FIG. 3.—Another multiplication rosette of short, narrow spindles. Whips as before.

FIG. 4.—Large multiplication rosette of fully developed spindles. Whips as before. Some cells dividing.

FIG. 5.—Small rosette from same preparation as preceding. The two rounded cells are in process of division as seen by the two stalks or whips which each gives off; also by the partial division of the blepharoplast.

PLATE 9.—CULTURAL FORMS OF *Tr. avium.*

From the same preparation as before. The figures show the initial stage of rosette formation.

FIG. 1.—The remnant of whip shows on each cell. The smaller cell has not begun to divide, the larger shows two nuclei and two blepharoplasts; one of the latter is dividing.

FIG. 2.—Large form as before, with four nuclei and five blepharoplasts.

FIG. 3.—Rosette of several such bodies.

FIG. 4.—Two cells resulting from the division of the round form.

FIG. 5.—Early stage of rosette consisting of four cells. Beside it a spirochete.

FIG. 6.—Rounded form, possibly of a spirochete, shows three nuclei and three blepharoplasts deeply stained. The lines on the surface are probably short flagella.

PLATE 10.—CULTURAL FORMS OF *Tr. avium.*

From same preparation as before. The figures show the swarming spirochete type.

FIG. 1.—A group of spirochetes.

FIG. 2.—A very long spirochete (50 μ) and a short one.

FIG. 3.—Agglutination of two spirochetes, showing the overlapping of the posterior ends.

FIG. 4.—Another agglutinated pair of spirochetes; the two cells are almost fused together.

A mass of agglutinated spirochetes is shown in Fig. 5, Plate 11.

PLATE 11.—CULTURAL FORMS OF *Tr. avium.*

From same preparation as before, except in case of Fig. 5.

FIG. 1.—Incomplete division of a spirochete; two separate nuclei while the dark oval blepharoplast shows that it is constricted, if not actually divided.

FIG. 2.—More advanced stage of division of spirochete, showing two nuclei and two blepharoplasts.

FIG. 3.—Complete division of a spirochete. The posterior end of one cell projects beyond that of the other.

FIG. 4.—Rounding up of a spirochete.

FIG. 5.—A mass of agglutinated spirochetes from strain Y. This was obtained from a canary inoculated with strain D. The preparation was living and motile. The photograph was taken by means of instantaneous exposure. Magnification, 500 ×.

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Reprinted from the American Journal of Physiology.
VOL. XIII. — JUNE 1, 1905. — NO. V.

THE PHYSIOLOGICAL BEHAVIOR OF METHYLENE BLUE AND METHYLENE AZURE: A CONTRIBUTION TO THE STUDY OF THE OXIDATION AND REDUCTION PROCESSES IN THE ANIMAL ORGANISM.

BY FRANK P. UNDERHILL[1] AND OLIVER E. CLOSSON.

[*From the Sheffield Laboratory of Physiological Chemistry, Yale University.*]

THE uses to which methylene blue has been put are manifold. Therapeutically, it has been employed for years as a bactericidal agent, and as a stain for tissues and bacteria it is one of the best reagents known. Because of its property of readily undergoing reduction it has proved an excellent agent for the study of the reduction processes of the animal organism. Its behavior in the body and its elimination have been the subject of many investigations, the more important of which have been carried out by Ehrlich,[2] Dreser,[3] Müller,[4] Elsner,[5] and others,[6] who have shown that, when introduced into the body, methylene blue is excreted in the urine and fæces in the form of methylene blue, its leuco compound, and one or more chromogenic substances.

Recently, Herter,[7] in a study of the reduction processes of the organism by means of methylene blue, observed the appearance in the liver of a substance eliminated in the urine and bile which, upon treatment with acid, heat, and an oxidizing agent, was transformed from a colorless to a blue compound. From the nature of this sub-

[1] Research Fellow of the Rockefeller Institute for Medical Research.

[2] EHRLICH: Centralblatt für die medicinischen Wissenschaften, 1885, xxiii, pp. 113 and 163.

[3] DRESER: Zeitschrift für Biologie, 1885, xxi, p. 41; *Ibid.*, 1886, xxii, p. 56.

[4] MÜLLER: Deutsches Archiv für klinische Medicin, 1899, lxiii, p. 130.

[5] ELSNER: *Ibid.*, 1901, lxix, p. 47.

[6] ACHARD and CASTAIGNE: Comptes rendus de la société de biologie, 1897, xlix, p. 1091; BRAUER: Zeitschrift für physiologische Chemie, 1903–04, xl, p. 182; HERTER: *Ibid.*, 1904, xlii, p. 403; also This journal, 1904, xii, pp. 128 and 207.

[7] HERTER: *Loc. cit.*

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stance, Herter was led to the opinion that methylene blue undergoes reduction to the leuco compound, which is in part excreted as a conjugate body, possibly a glycuronic acid compound. At the suggestion of Professor Herter, the nature of this "paired body" has been made the subject of the present investigation.

With What Does Methylene Blue Conjugate?

Various investigators have shown that when dilute solutions of methylene blue are slowly injected into the circulation of a rabbit, the dye is reduced to the leuco compound. The tissues appear normal when first exposed to the air, but soon undergo a change whereby they assume a blue-green color; or, if an oxidizing agent, such as hydrogen peroxide, is applied to the tissues, the change takes place immediately, the leuco compound being oxidized to the blue salt. According to Herter, it is possible to regain only part of the introduced dye in this way. In the liver, bile, and urine there is found a water-soluble compound, the "paired body," which does not become blue spontaneously or on boiling in the presence of an oxidizing agent, but which upon heating with an acid, like hydrochloric or acetic, grows intensely blue.

Preliminary experiments with rabbits which had received injections of methylene blue under conditions in every way comparable with those in Herter's investigation, yielded results in apparent agreement with them. For instance, after the slow injection of Grübler's methylene blue, the urine and bile, on boiling with acid and subsequent oxidation with hydrogen peroxide, assumed a deeper blue coloration than on oxidation alone. The liver extract behaved in a similar manner. Among the substances that most commonly unite with foreign compounds when introduced into the organism, are glycuronic and sulphuric acids, glycocoll and cholic acid. In order to show the presence or absence of a conjugate body formed by one of these compounds and methylene blue, the urine of a dog of 6 kgm. that had received an intraperitoneal injection of 50 c.c. of a 1 per cent solution of Grübler's methylene blue was studied. Search was made for conjugated glycuronates and glycocoll compounds with negative results. Three days after the injection of the methylene blue, the greater portion of the dye had been excreted, so that the urine for the two days following was of nearly normal color. In such a urine, color changes can be noted very readily. A study of these urines brought out the

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following facts: acidification of the urine caused no change of color, but upon boiling an acidified urine the appearance of a pronounced green color was noted. If the urine was boiled for a period of three to five minutes without addition of acid, a result in every way similar was obtained. On treatment of the urine with an oxidizing agent (hydrogen peroxide), whether in acid or neutral solution, there was no immediate change of color; but when the solution was allowed to stand for a period of two hours, a green color was observed which was in every way comparable with that caused by boiling an acidified urine. If the urine, as excreted, stood in contact with the air for two hours, a decided green color developed.

From the foregoing observations it would seem that if a "paired body" is present in the urine after methylene blue injections, the conjugation cannot be of a very stable nature, inasmuch as the combination can be broken up by boiling. Such substances as the conjugated sulphates, glycuronates, etc., require far more vigorous treatment in order to effect the cleavage into the constituent bodies.

If the tissues (liver, muscles, and kidneys) of a rabbit that has received an injection of methylene blue (0.33 per cent solution) are ground with sand and repeatedly extracted with warm water a point can be reached after several days when the tissues become free from foreign coloring matter. Boiling the extracted tissues or their respective extracts in an acid solution produces no deepening of color. The extraction of the dye from the tissues is much more rapid when a faintly acid solution is employed than when water is used. The acid seems also to intensify the color of the extracts. So far as the tissues are concerned it would appear that if any conjugation with methylene blue does occur, it is in all probability of the nature of a combination between the cell protoplasm and methylene blue similar to that found by Mathews[1] and Heidenhain[2] for many dyes. According to these investigators, the proteids may be regarded as possessing both acid and basic properties. If a dye, like methylene blue, which is a salt, is added to an alkaline proteid solution, the base of the dye is set free and a union effected with the proteid. It can be readily seen how such a combination may be broken up by simple boiling, and why acid might be a much better solvent for the dye than water.

In view of the foregoing facts, the conclusion seems warranted that

[1] MATHEWS: This journal, 1898, i, p. 445.

[2] HEIDENHAIN: Archiv für die gesammte Physiologie, 1902, lxc, p. 113, and 1903, lxcvi, p. 440.

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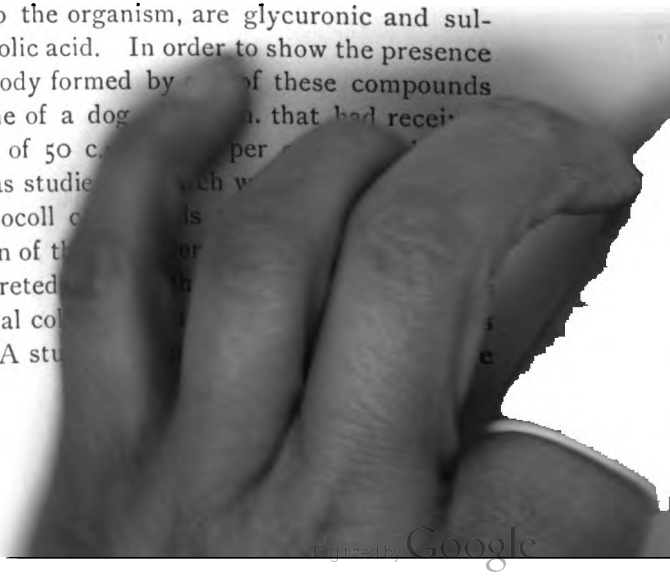

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[1] MATHEWS: This journal, 1898.

[2] HEIDENHAIN: Archiv für die ge
1903, lxcvi, p. 440.

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the evidence of a "paired body"[1] in the organism after injections of methylene blue is insufficient.

The Excretion of Methylene Blue.

In the course of the observations on dog's urine excreted after the injection of methylene blue, it was noted that if such a urine is made faintly ammoniacal and extracted with ether (or chloroform), the ether assumed a beautiful red color, while the urine regained its normal appearance. The addition of a few drops of acid to the ethereal solution caused the formation of two layers of liquid, the upper (ethereal) becoming colorless, while the lower turned an intense blue or blue-green. Again making the ethereal solution ammoniacal, and shaking, led to the reappearance of the red color in the ether, and to the decolorization of the aqueous layer. Distillation of the ether from the red ethereal solution resulted in a blue residue, regardless of the reaction of the fluid. An examination of this coloring matter showed that it presented an absorption spectrum differing slightly from that given by methylene blue. In ethereal solution it yields no absorption bands. Similar results could be obtained with aqueous extracts of the several tissues of animals that had received methylene blue injections. A large quantity of the same substance could be demonstrated in the commercial methylene blue employed for the injections.

A study of the chemistry[2] of methylene blue has shown that the methylene blue preparations available (Grübler's, Kahlbaum's, crystalline, and Merck's, medicinal) are mixtures of methylene blue and methylene azure. Methylene azure is formed from methylene blue by treatment with strong alkalies, and is a contamination which it is almost impossible to separate from methylene blue. Like the latter, it may exist in three forms: (*a*) the free base, (*b*) a salt, (*c*) the leuco compound. The latter is formed by reducing agents with as great ease as the leuco methylene blue is formed from its precursor. The free base of methylene blue is blue and insoluble in ether. The free base of methylene azure is red and soluble in ether and chloroform. It has a great affinity for acid, since simply pouring an acid

[1] The idea of a conjugation after methylene blue injections is by no means new. Dreser (*Loc. cit.*) put forth the same view in 1885, but it could not be confirmed by Ehrlich (*Loc. cit.*).

[2] For the chemistry of methylene blue, see Bernthsen: Liebig's Annalen der Chemie, 1885, ccxxx, p. 73; and 1899, ccli, p. 1.

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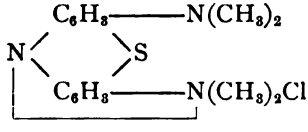
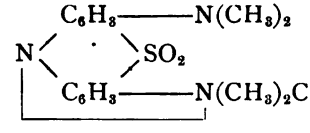
into an ethereal solution will render the ether colorless, while the acid becomes blue, owing to the formation of a salt which is blue. This blue salt has properties in every way identical with methylene blue, with the single exception of its spectrum. In strong solutions, methylene blue gives a spectrum with two absorption bands, the broader one, between *B* and *C*, the other between *C* and *D*. In dilute solutions, only the more intense band may show. In strong solutions, methylene azure also has two absorption bands, but both are between *C* and *D*. Only one band appears in dilute solutions. The salts of both methylene blue and methylene azure are insoluble in ether. The salt of methylene blue is slightly soluble in chloroform, while the leuco compounds of both dyes are insoluble in alkalies and water.

That methylene azure is a contamination originally present, and is not formed from methylene blue by the slight addition of ammonia, may be seen from the following experiment. Methylene blue (Kahlbaum, crystalline) was dissolved in water, made faintly ammoniacal, and shaken with ether until the ether was no longer tinged with red. The purified aqueous solution was then made distinctly ammoniacal, and allowed to stand for one hour. At the end of this time no methylene azure could be detected. After a period of twelve hours, however, a noticeable quantity of methylene azure had been formed. While several volumes of concentrated ammonia are necessary to rapidly convert a portion of the methylene blue into the azure compound, dilute ammonia will tend, on long standing, to accomplish the same action.[1] The quantity shaken out by a faintly ammoniacal ethereal solution cannot, however, be accounted for by the action of the ammonia upon the methylene blue. Addition of ammonia, in the quantities employed here, transforms the methylene azure from the salt to its free base form. Methylene azure differs in composition from methylene blue only by containing two atoms of oxygen. Thus:



Methylene blue(salt)



Methylene azure (salt)

The transformation of methylene blue to methylene azure appears to be very easily effected. It is unknown whether the reverse change can take place.

[1] *Cf.* BERNTHSEN: *Loc. cit.*, 1885, p. 169.

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In order to study the behavior of methylene blue in the body, it is obviously desirable that the dye be in a form uncontaminated. Accordingly, experiments have been carried out with this purpose in view. The results show that the only satisfactory method of purification which gives a methylene blue solution free from methylene azure is the following: solutions of methylene blue are made distinctly ammoniacal and shaken with ether until the latter is no longer colored red. The process is long (one to two days, according to the strength of the solution) and requires large volumes of ether. After the methylene azure has been removed, the methylene blue solution is made acid with hydrochloric acid, and concentrated to a smaller volume. On cooling, the methylene blue salt crystallizes in long needles. Acid solutions of methylene blue remain unchanged for indefinite periods of time, but alkaline solutions, on standing for some hours, readily give evidences of the presence of methylene azure, and after three weeks such solutions yield large quantities of the azure compound.

The behavior of pure methylene blue obtained by the method outlined above has been studied in the organism of man, dog, cat, and rabbit. Typical protocols follow:

Experiment 3. — A rabbit of 3 kgm. under ether anæsthesia, received an intravenous injection of 87 c.c. of a 0.5 per cent solution of *pure methylene blue* in 55 minutes. The tissues presented the appearance usual after methylene blue injection, that is, they rapidly changed from the normal color to a blue or blue-green hue. On addition of hydrogen peroxide or acetic acid, an intense blue coloration developed immediately. The urine and bile, when made ammoniacal and shaken with ether, gave evidence of the presence of both methylene blue and methylene azure. Only traces of methylene azure were noted in the urine; while in the bile, methylene azure could be shown in much greater quantity than methylene blue. The muscles, liver, stomach, and kidneys were ground finely and extracted with warm water until all foreign coloring matter had been taken out. All of the aqueous extracts were found to contain a little of the free methylene azure base. This was ascertained by shaking the extract with ether, whereupon the ether became tinged with red. A small quantity of methylene azure, and a much larger amount of methylene blue, was present. Boiling the extracted tissues and the aqueous extracts with acid did not cause a change of color.

Experiment 4. — 10 c.c. of a 0.35 per cent solution *pure methylene blue* were intravenously injected into a rabbit of 1200 gm. and under ether anæsthesia. Examination of the urine of the following day revealed the

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presence of relatively large quantities of methylene azure and only traces of methylene blue. The second day following the injection, traces of methylene azure only could be found. The urine on the third day was normal.

Experiment 5. — To a cat of 2.5 kgm. were administered intravenously 10 c.c. of a 0.25 per cent solution of *pure methylene blue.* No urine was excreted until the third day after the injection. This urine was perfectly normal in appearance, and gave no evidence of chromogenic substances on oxidation, or on boiling after acidification.

Experiment 6. — A cat of 3 kgm. received an intravenous injection of 20 c.c. of a 0.35 per cent *pure methylene blue* solution. The next day no urine was excreted. On the second day the urine contained much methylene blue and significant quantities of methylene azure. Fæces passed on this day also contained traces of both substances.

Experiment 7. — A dog of 8.5 kgm. received an intraperitoneal injection of 20 c.c. of a 0.35 per cent solution of *pure methylene blue.* The urine of the next day contained both methylene blue and methylene azure.

In order to test the behavior of methylene blue in the organism of man, Merck's medicinal methylene blue was purified in the manner previously described. A typical experiment follows:

Experiment 8. — To three subjects, A, B, C, *pure methylene blue* was administered in capsules containing doses of 75, 50, and 75 mgm. respectively. One and one-half hours later the urines of all three subjects contained significant quantities of methylene azure and larger amounts of methylene blue. Two days later all the methylene blue had disappeared, but a trace of methylene azure could be shown. Both compounds were found in small amounts in the fæces.

A review of the experiments outlined above shows that pure methylene blue, whether administered intravenously (cat and rabbit), intraperitoneally (dog), or by way of the mouth (man), in relatively large doses is eliminated by the kidneys and intestines in the same forms, that is, as methylene blue and methylene azure. The presence of the latter body in the urine and fæces is unquestionably due to the oxidative changes going forward in the organism, whereby the methylene blue is in part oxidized to methylene azure. When pure methylene blue is injected intravenously in *small* doses (10 c.c. of a 0.25 per cent solution), in the cat at least, the dye, or its known decomposition products, fails to appear in the urine or fæces.

If the freshly voided urine of an animal that has received an injec-

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tion of methylene blue is boiled for several minutes, the blue coloration is intensified, although another portion of the same urine does not show any deepening of color on treatment with an oxidizing agent. Acidification of the urine with subsequent boiling causes the blue coloration to deepen in degree equal to that caused by several minutes boiling without addition of acid. The depths of color in the two cases are not to be distinguished from one another. If the urine is vigorously and repeatedly extracted with ether, the color changes of the urine noted after boiling and acidification and boiling no longer appear. The ethereal extract remains colorless. When the latter is treated with acetic acid, there is a formation of two layers of liquid, the upper, ethereal layer remaining colorless, the lower, acid layer becoming deep blue. Made ammoniacal and shaken, the ethereal solution becomes red, while the alkaline fluid is blue. Spectroscopic examination of these two solutions after acidification shows that the red ethereal layer is a solution of methylene azure, and that the blue ammoniacal solution consists of methylene blue. From these results it would seem that methylene blue is excreted also in the forms of the leuco compounds of both methylene blue and methylene azure. Additional experiments bear out this conclusion. A portion of the colorless ethereal extract of an experimental urine treated with water, and vigorously shaken to give large surface exposure to the air, gradually assumed a deep blue color, which could be shown to consist of a mixture of methylene blue and methylene azure. Addition of water to a second portion, without shaking, did not cause a development of color on standing for one hour. From another portion, the ether was evaporated, and the blue residue, when taken up in water, was found to consist of a mixture of methylene blue and methylene azure. Addition of hydrogen peroxide to a fourth portion of the ethereal extract caused the formation of a blue solution.

From the experimental observations noted, the conclusion seems justified that when methylene blue is introduced into the animal organism, it is excreted in at least four forms: namely, methylene blue, methylene azure, and their corresponding leuco compounds. The two latter substances are not oxidized so readily as one would expect from a perusal of the literature on the subject, as may be seen from the following experiment. 1. A solution of pure methylene blue is completely decolorized by ammonium sulphide, and is divided into two portions. One portion very rapidly turns blue on exposure to the air, while the other assumes an intense blue color when treated

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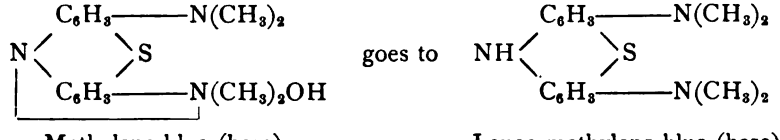
with hydrogen peroxide. 2. A pure methylene blue solution is completely decolorized with ammonium sulphide, and at once made acid with acetic acid, and then divided into five portions. The first portion is allowed to stand in a shallow dish exposed to the air. After a period of twenty-five to thirty minutes, the colorless solution begins to assume a blue coloration, which very gradually deepens. Hydrogen peroxide added to a second portion causes a development of color after fifteen to twenty minutes. Through the third portion a current of air is passed, to expel the hydrogen sulphide gas. The appearance of a blue color is not to be observed until fifteen to twenty minutes after the expulsion of all the hydrogen sulphide gas, and then the color development is extremely slow. If the fourth portion is boiled for one to two minutes, the solution becomes intensely blue. If to the fifth portion alkali is added until the reaction is alkaline, the blue color returns at once. Here it will be noted that the results found are identical with those that obtain in a methylene blue urine; for if such a urine is boiled for two to three minutes, it deepens in color. Addition of acid, with subsequent heating, causes the development of the blue color more rapidly. Standing in the air will cause the methylene blue urine to assume gradually a deeper hue, as will addition of hydrogen peroxide and standing for a certain length of time.

What is the explanation for the difference in behavior between an alkaline and an acid solution of the reduced methylene blue? Ordinary methylene blue is a salt. When ammonium sulphide is added to a methylene blue solution, two things happen: namely, the alkali transforms methylene blue (salt) into methylene blue (base), thus:

Methylene blue (salt) Methylene blue (base)

while the latter is reduced to the leuco compound, also a base, thus:



Methylene blue (base) Leuco methylene blue (base)

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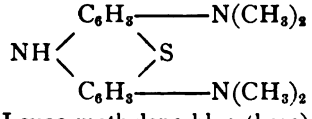
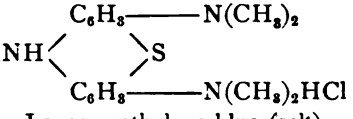
so that the solution contains the leuco compound of methylene blue which is a base. Standing in the air, or treatment with an oxidizing agent, causes this substance to be quickly changed to the colored compound — the methylene blue base. If, however, the solution of the leuco methylene blue base is made faintly acid, the oxidation does not ensue so readily.[1] A new compound, less easily oxidized, has been formed. Represented graphically, the change would be as follows:



Leuco methylene blue (base)



Leuco methylene blue (salt)

It is this leuco compound, the salt,[2] which is the substance probably present in the urine. It is the reaction of the medium containing the leuco body which is the essential factor; for when the solutions are alkaline, the oxidation of the leuco compound is rapid, whereas blue color develops very slowly in the presence of acid. Observations similar in every respect can be made with methylene azure. The weaker the acid medium, the more striking are the results that may be obtained. Acid potassium phosphate acts much better than acetic acid, and an acetic acid medium is much more favorable than one made acid with hydrochloric acid. Here the results obtained with a methylene blue solution are identical with those that may be observed in a methylene blue urine. The objection might be offered that when a colorless ammonium sulphide solution of the methylene blue compound is made acid, sufficient hydrogen sulphide is present to prevent the oxidation, that is, hydrogen sulphide will still function as a reducing agent, and hence the solution will remain colorless. Two observations speak against such an argument. In the first place, even though there is a large excess of hydrogen sulphide present in such a solution, addition of an alkali causes the immediate appearance of the intense blue coloration. Secondly, when the hydrogen sulphide is removed by a current of air, the solution does not become blue until many minutes have elapsed.

This behavior of methylene blue and its derivatives is sufficient to account for the reactions attributed by Herter to a "paired sub-

[1] *Cf.* BERNTHSEN: *Loc. cit.*, 1885, p. 149.

[2] Regarding the existence of such a compound, see BERNTHSEN: *Loc. cit.*, 1885, p. 150.

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stance." The presence of the four substances, methylene blue methylene azure, leuco methylene blue (salt), and leuco methylene azure (salt), has been demonstrated in the urine of the dog, cat, rabbit, and man, after the introduction of pure methylene blue. In the aqueous extracts of the liver, muscles, kidneys, and bile of the rabbit, similar compounds may also be shown after injections of pure methylene blue.

A single experiment was performed to determine whether the methylene blue was absorbed into the lymphatic system. The protocol follows.

Experiment 9. — Lymph was collected from the thoracic and cervical ducts of a dog of 20 kgm. under morphine and A. C. E. anæsthesia. The urine was obtained from cannulas in the ureters, and the bile from a biliary fistula. 200 c.c. of crude methylene blue solution were injected into the facial vein of the animal in two hours and thirty minutes. One hour and twenty minutes after the beginning of the injection, the urine was colored blue, and twenty minutes later the bile contained the dye. The cervical lymph remained free from coloring matter, and in the lymph from the thoracic duct, the presence of chromogenic substance was doubtful. This lymph was somewhat tinged with blood, and yielded no more color on oxidation than might be expected from slight contamination with blood containing the leuco compounds. On oxidation, none of the organs, except the kidney, showed the least trace of methylene blue. Treatment of this tissue with an oxidizing agent transferred the intensity of the blue color from the medulla to the cortex. The blood of the animal had not clotted thirty-six hours after death.

It appears in this case at least that the methylene blue is transformed or excreted before it is distributed at all widely in the lymph spaces. Trials were also carried out to determine whether isolated surviving organs, such as the liver, kidney, and spleen are capable of transforming pure methylene blue to methylene azure. The results were negative.

The Excretion of Methylene Azure.

It has been shown above that methylene blue is in part transformed into methylene azure when introduced into the body. The behavior of the latter substance in the organism has been made the subject of further study.

Spectroscopic examination of a methylene azure preparation labelled

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"Methylene azure hydrochloride, puriss,"[1] indicated the presence of a relatively large quantity of methylene blue. Accordingly, pure methylene azure was prepared from impure methylene blue in the following way. A strong solution of the dye was treated with strong ammonia, and extracted with ether in a Kutscher-Steudel apparatus for several days. The extract was freed from ether and ammonia by heating, and the residue extracted with water. The methylene azure in the aqueous solution was shaken out with ether, and the latter removed by distillation. A 0.05 per cent solution was used for the injections. Three typical experiments are appended.

Experiment 10. — Into a rabbit of 1200 gm. were injected intravenously 90 c.c. of a 0.05 per cent solution of *pure methylene azure* in fifty minutes. Twelve minutes later the animal was killed by bleeding. The blood serum contained a trace of methylene azure. The tissues appeared normal and did not reveal a trace of blue coloration after treatment with hydrogen peroxide or acetic acid. The bile contained a little methylene azure, while the urine was free from it. The only organ or tissue that gave evidence of foreign coloring matter was the stomach with its contents. Of the aqueous extracts of the liver, muscles, kidneys, small and large intestines, and stomach examined, only the liver and stomach revealed the presence of a dye; here only traces of methylene azure and its leuco compound.

Experiment 11. — A rabbit of 2.5 kgm. received an intravenous injection of 20 c.c. of a 0.15 per cent solution of *pure methylene azure* in fifteen minutes. After ten minutes the animal was killed. A trace of methylene azure was detected in the blood serum. The tissues appeared normal; but upon treatment with acetic acid or hydrogen peroxide, assumed a green coloration that was just discernible. A trace of leuco methylene azure and methylene azure could be demonstrated in an aqueous extract of the muscles. The urine contained methylene azure and leuco methylene azure (salt), and behaved toward acid and oxidizing agents in a manner similar to a methylene blue urine.

Experiment 12. — 30 c.c. of a 0.15 per cent solution of *pure methylene azure* were administered intraperitoneally to a dog of 10 kgm. The urine as well as the fæces of the following two days contained methylene azure and the leuco compound. Subsequently the urine contained no colored bodies. The total quantity recovered was decidedly smaller than that injected.

Experiment 13. — After the intravenous injection of 10 c.c. of the same solution of methylene azure into a cat of 3 kgm. no trace of the dye could be detected in the urine or fæces.

[1] Kindly furnished by Professor HERTER.

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Such experiments demonstrate that when methylene azure is introduced intravenously (in the rabbit and cat) or intraperitoneally (in the dog) it reappears in the urine and fæces in part as the unchanged dye and partly as a leuco compound. Both forms are found in the tissues. Furthermore, when a small enough dose is given, none can be regained from the urine or fæces. Even after a large dose, the quantity excreted is far less than that introduced. The major portion does not reappear, at least in a chromogenic form. From this it may be concluded that the dye has undergone a change, probably oxidative in character, whereby the methylene azure is transformed into one or more decomposition products, the nature of which is at present unknown.

Elsner[1] has studied the excretion of methylene blue quantitatively, and has shown that at most only 50 to 60 per cent of the introduced dye can be regained in the urine and fæces. Müller[2] found that methylene blue is eliminated from the animal organism in at least four forms, namely, methylene blue, an unknown oxidation product of the dye, and two unknown chromogenic substances. The present confirmatory experiments afford an explanation of the results of both investigators. The unknown oxidation product of Müller is in all probability methylene azure, and the leuco compounds of the writers are probably identical with Müller's unknown chromogenic substances. The explanation of Elsner's results lies in the observation that methylene blue may in part be transformed to methylene azure, and that the latter is partly decomposed, and does not reappear as a chromogenic substance.

The results obtained *in vivo* may be imitated in test-tube experiments. Solutions of *pure methylene blue* heated with dextrose undergo no change. If a drop of potassium hydroxide is added to the hot solution, two reactions occur, — the solution is decolorized (leuco methylene blue (base) is formed and separates), and a significant quantity of methylene azure (in the leuco form) arises. Boiling the methylene blue solution in the presence of potassium hydroxide causes the formation of the methylene azure without the development of any reduction product. Ammonia substituted for potassium hydroxide does not lead to a formation of methylene azure, although the reduction takes place. Here the alkali seems to be the effective factor; possibly in the organism the transformation of methylene blue into

[1] ELSNER: *Loc. cit.*

[2] MÜLLER: *Loc. cit.*

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its oxidation product, methylene azure, is facilitated or inaugurated by the presence in the tissues of traces of alkaline salts.[1] The products formed suggest that the processes involved are essentially those of oxidation and reduction, which may possibly occur simultaneously in the same tissue or tissues. Thus the fate of methylene blue demonstrates not only the reduction processes of the body, but also those of oxidation, and presumably the conditions that modify the former also exert an influence on the latter.

Summary.

The evidence offered of a conjugate body formed in the organism after the introduction of methylene blue is insufficient. The experimental facts can be accounted for by the presence in significant quantities of two chromogenic substances, leuco compounds corresponding to methylene blue and methylene azure.

The introduction of pure methylene blue, whether intravenously, intraperitoneally, or by way of the mouth, is followed by the appearance in the urine and fæces of methylene blue, methylene azure, — an oxidation product of methylene blue, — as well as leuco compounds corresponding to methylene blue and methylene azure.

When pure methylene azure is injected, intravenously or intraperitoneally, only a portion can be regained, consisting of methylene azure and a leuco compound of this dye. Small doses of either methylene blue or methylene azure fail to reappear in the urine. It is only when the oxidative processes of the organism are inadequate that these bodies are manifested. The present experiments therefore demonstrate the simultaneous action of both oxidation and reduction processes in the animal organism.

[1] If a solution of crude methylene blue is treated with calcium dioxide, or magnesium dioxide, and tartaric acid, the depth of color of the solution is markedly decreased. Examination shows that the greater portion of both methylene blue and methylene azure present has been oxidized to colorless substances. Hydrogen peroxide fails to call forth this reaction.

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[Reprinted from THE MEDICAL NEWS, June 17, 1905.]

ON THE OCCURRENCE OF SPIROCHAETA PALLIDA, SCHAUDINN, IN SYPHILIS.[1]

BY SIMON FLEXNER, M.D. AND HIDEYO NOGUCHI, M.D.,
OF NEW YORK.

IN the last few weeks several papers have appeared in the German and French medical literature,[2] dealing with the spirochaete which Schaudinn and Hoffmann described as occurring in recent syphilitic lesions. The first paper by Schaudinn and Hoffman appeared almost simultaneously in the Arbeiten aus dem Kaiserlichen Gesundheitsamte and the *Deutsche medicinische Wochenschrift*. The paper in the former publication was illustrated with two microphotographs, showing the form of the organism. These papers were quickly followed by two communications from Metchnikoff's laboratory in the Pasteur Institute, confirming and accepting the discovery, and drawing attention to the interesting fact that Bordet and Gengou had observed the same micro-organism in a syphilitic chancre some three years before. However, as they failed to discover it in some syphilitic lesions which they subsequently studied, they abandoned any future search for it.

Hoffmann and Schaudinn's first publication dealt with a study of primary chancres, the enlarged glands of the groin attending these lesions and flat condylomata in syphilitic patients. The study consisted in the examination of fresh specimens obtained from the surface and interior of the primary lesions and the interior of lymph glands and condylomata, and stained specimens from the same sources. Certain control examinations were also made of non-syphilitic lesions of the genitals and of mixed lesions of these parts. The results were quite uniform and suggestive. From the cases of simple syphilitic infection the lymph glands, condylomata and interior of chancres, showed a variable number of spiral micro-organisms of great tenuity for which they propose tentatively the name *Spirochaeta pallida*, while the non-specific lesions showed a second spiral

1 From the Rockefeller Institute for Medical Research, New York.

2 The complete bibliography of the subject, up to the present time, is as follows:

Schaudinn and Hoffmann. Vorläufiger Bericht über das Vorkommen von Spirochaeten in syphilitischen Krankheitsprodukten und bei Papillomen. Arbeiten aus dem Kaiserlichen Gesundheitsamte. Berlin, April 10, 1905, XXII, Zweites Heft, 527.

Schaudinn and Hoffmann. Ueber Spirochaetenbefunde im Lymphdrüsensaft Syphilitischer. Deutsche medizinische Wochenschrift. May 4, 1905, XXXI, 711.

Metchnikoff and Roux. Recherches microbiologiques sur la syphilis. Bulletin de l'Académie de Medicine. Paris, May 16, 1905.

Buschke and Fischer. Ueber das Vorkommen von Spirochaeten in inneren Organen eines syphilitischen Kindes. Deutsche medizinische Wochenschrift, May 18, 1905, XXXI, 791.

Levaditi. Syphilis congenitale et Spirochaeta pallida Schaudinn, Comptes rendus de la Société de Biologie. May 26, 1905, LVIII, 845. La Presse Médicale, May 31, 1905, No. 43, 337.

Schaudinn and Hoffmann. Ueber Spirochaeta pallida bei Syphilis und die Unterschiede dieser Form gegenüber anderen Arten dieser Gattung. Berliner klinische Wochenschrift, May 29, 1905, XLII, 653.

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micro-organism, for which they also propose the name of *Spirochaeta refringens*. The latter organism had, doubtless, been seen and described before by several observers, notably by Berdal and Bataille,[1] Csillag[2] and Rona.[3] Schaudinn and Hoffmann did not find the first spirochæte in non-syphilitic lesions, nor did they find the second in the interior of the syphilitic lesions studied by them. From the study of Schaudinn and Hoffmann it is not difficult to explain the failure of previous investigators to perceive *Spirochaeta pallida* and in especial Bordet and Gengou's failure to obtain it in all of the several cases studied by them. The organism is difficult to see in the fresh state, and it is also highly refractory to staining so that special methods are required to demonstrate it in fixed preparations. The description of the organism is as follows:[4] In the length the spirochaeta varies from 4 to 10 μ, the average being 7 μ; in width the variation is from unmeasurable thinness to $\frac{1}{2}$ μ. The number of bends is from 3 to 12. The organism agrees in motility with the spirochaetæ rather than the spirilla; there are three characteristic movements: rotation on the long axis, forward and backward motion and bending of the entire body. There are indications of an undulating membrane but none of flagella. The poles end in sharp points. No further details of structure have been made out thus far.

For the purpose of the study of the fresh material dilution with salt solution of the expressed juices of primary lesions, or the fluid drawn by aspiration from the lymph glands, is permissible. Prepared in this way the spirochaeta were still actively motile, according to Schaudinn and Hoffmann, after six hours.

The staining is accomplished with difficulty and the best results thus far have been obtained with Giemsa's eosin solution and azure. Schaudinn and Hoffmann recommend the following formula:

Twelve parts of Giemsa's eosin solution (2.5 c.c. 1 per cent. eosin, 500 c.c. water).

Three parts Azur No. I (1:1,000 solution in water).

Three parts Azur No. II (0.8:1,000 solution in water).

The mixture is to be freshly prepared. The films, which should be thinly spread, are dried in the air and then hardened in absolute alcohol for ten minutes, after which they are immersed in the stain from sixteen to twenty-four hours. They are to be washed in water, dried in the air and examined in cedar oil.

Our study has thus far comprised a small nmber of recent cases of syphilis and two cases of non-syphilitic ulcer of the penis. We are indebted for the material studied to Dr. V. C. Pedersen, to whom we wish to express our obligations. The cases are as follows:

1 Quoted by Schaudinn and Hoffmann.
2 Quoted by Schaudinn and Hoffmann.
3 Quoted by Schaudinn and Hoffmann.
4 Schaudinn and Hoffmann. Arbeiten aus dem kaiserlichen Gesundheitsamte, 1905, XXII, zweites Heft, 527.

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Case I.—Male, twenty years old. Luetic infection December, 1904. No regular treatment. He presented mucous patches of the tonsils and soft palate, and a fading rash of the trunk. Between the buttocks, flat condylomata. A condyloma was excised. Smears were made and stained in various anilines and with eosin-azur. Fresh preparations in salt solution were also studied. In the latter no characteristic organisms were found. The stained preparations were positive, showing a variable number of thin, lightly-stained spiral organisms identified as the species described by Schaudinn and Hoffmann. The positive results were obtained with aniline-water-gentian violet and eosin-azur, the latter having given the most satisfactory results. The films varied greatly, even with the same method of preparation. In some the number of stained spirals was very small, while in others the number was large, a single field showing as many as five. In still other cover-glasses no organisms could be discovered. The spirals were long for the most part and showed from six to twelve bends or curves.

Case II.—Male, aged thirty-four years. Burrowing non-syphilitic ulcer of the penis. Smears from the surface only could be obtained and they were negative for spiral micro-organisms.

Case III.—Male, aged twenty-three years. Mucous patches in mouth. Healed scar on penis. Enlargement and induration of glands of the groin. A small quantity of fluid, consisting of blood and lymph cells aspirated from the enlarged glands. Fresh preparations negative; smears stained in eosin-azur showed a very few delicate, faintly-staining spiral organisms.

Case IV.—Male, aged eighteen years. Infection in January. For several weeks active antisyphilitic treatment. Primary lesion on glans penis; marked swelling of the glands of the groin. Part of primary lesion was excised and fluid was aspirated from an enlarged gland. In the fresh fluid of the gland diluted in sterile salt solution several small and a single larger motile spiral organism were seen. None of the stained preparations from the primary lesion or the gland juice showed spirochaetae.

Case V.—Colored male, aged twenty years. No definite history of time of infection. Presented himself with phimosis and balanitis. After incision of the prepuce three separate elevated, indurated lesions regarded as chancres appear. Moderate swelling of the glands of the groin. One of the lesions excised. Smears and fresh preparations were made from the base of the lesion. Fresh preparations negative. The smears stained in eosin-azur showed a moderate number of delicate spirals, agreeing with the description given by Schaudinn and Hoffmann. They varied in number in different cover glasses, and in some could not be found. The curves numbered from eight to twelve.

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Case VI.—Male, aged twenty-three years. Non-syphilitic ulcer of penis; duration twelve days. Films stained in eosin-azur were negative for spirochaetae.

From this small series of cases it will be seen that of the four cases of syphilitic lesions the spiral organisms were obtained in stained preparations three times, while in the two cases of non-syphilitic lesions studied they could not be found. An anomaly exists in respect to Case IV, in which the spirals were missed in stained preparations, while they appeared to be present in the fresh state. No explanation can now be offered for this occurrence.

Schaudinn and Hoffmann express themselves very guardedly regarding the significance of the spirochaeta. They point out its presence in the typical lesions of the disease and its absence in the other forms of venereal disease studied. Important confirmatory contributions have come from Metchnikoff and Roux, who have demonstrated the same organism in the lesions of acquired syphilis in man and in experimental syphilis in the monkey and ape. In the last animals the material for study was obtained from the primary lesions produced by inoculation before ulceration had taken place. Additional confirmatory evidence of importance as regards the distribution of the spirochaeta is supplied by the observation of Levaditi and Buschke and Fischer upon congenital syphilis. These writers found that organism in the pemphigus bullæ and papules of the skin, and, in cases coming to autopsy, in films from the spleen and liver. Schaudinn reports that he also has obtained it from splenic juice removed by aspiration from a syphilitic patient.

Metchnikoff and Roux draw attention to the irregularity of distribution of the organism as indicated by the variation in numbers upon the cover slips. We have observed the same irregularity, but we are not yet prepared to state that the difference may not be due to the imperfect technic in staining. Metchnikoff and Roux and Levaditi prefer a more rapid method of staining the films, namely that of Marino,[1] which, up to the present, we have used but little. Should it serve as good a purpose as the slower eosin-azur, and should future study confirm the etiological position of the spirochaeta, a rapid and useful, and perhaps even a specific method of diagnosis would be afforded. Since the organism exists in the primary lesions and the glands of the groin in a demonstrable form, and since fluid from each can be obtained easily, with the infliction of little pain to the patient, and without in any way prejudicing the progress of his disease, we may look for a general study of the fluids obtained from these sources in suspicious and established forms of venereal disease with reference both to the occurrence and the specificity of *Spirochaeta pallida.*

1 Annales de l'Institut Pasteur, 1903, XVII, 362.

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Darstellung und Analyse einiger Nucleinsäuren.

VIII. Mitteilung.

Über die Milznucleinsäure.

Von

P. A. Levene.

(Aus dem Rockefeller Institute for Medical Research.)

(Der Redaktion zugegangen am 19. Juni 1905.)

Bei der Analyse verschiedener Nucleinsäuren, soweit sie bis jetzt beschrieben sind, richtete der Verfasser seine Aufmerksamkeit auf das Studium der Komponenten ihrer Moleküle.[1]) Eine quantitative Schätzung der Bestandteile konnte deshalb nicht vorgenommen werden, weil von den beiden Darstellungsmethoden der Säuren, die eine die Substanz nicht in hinreichender Menge lieferte, die andere aber ein Produkt, das nicht völlig frei von Verunreinigungen war.

Im Verlaufe dieser Untersuchungen wurde nun die Beobachtung gemacht, daß Nucleinsäuren in großem Überschuß von konzentrierter Essigsäure löslich sind, und aus solchen Lösungen frei von den gewöhnlichen Verunreinigungen durch Kupferchlorid oder Salzsäure gefällt werden. Die Ausbeute war außerdem ganz zufriedenstellend und so wurde es möglich, die Frage der quantitativen Bestimmung der basischen Komponenten der Säuren in Angriff zu nehmen. In dieser Publikation sollen die Resultate der Analyse von Milznucleinsäure mitgeteilt werden.

Die Darstellung der Säure war folgende:

Die zerkleinerten Drüsen wurden ungefähr 2 Stunden in einer 5%igen Kochsalzlösung gekocht, worauf 7% des Gewichtes

[1]) Diese Zeitschrift, Bd. XXXII, S. 541; Bd. XXXI, S. 402; Bd. XXXVII, S. 402; Bd. XXXIX, S. 4; Bd. XXXIX, S. 479.

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der frischen Drüse an Natriumacetat hinzugefügt wurde und die Mischung abkühlte. Darauf erfolgte Zusatz von Natronlauge und die so behandelten Drüsen wurden über Nacht stehen gelassen. Wie in früheren Mitteilungen beschrieben, wurden nun die Eiweißkörper der Drüse mit Pikrin- und Essigsäure entfernt, und im Filtrat die Nucleinsäure mit Alkohol gefällt. Nachdem die Alkoholfällung mit Natronlauge wieder aufgelöst war, wurde ein großer Überschuß von Eisessig hinzugefügt und abgesaugt. Hierauf erfolgte Fällung der Nucleinsäure mit 20%iger Kupferchloridlösung, dann Waschen und Trocknen in gewöhnlicher Weise.

Die Analyse der auf diese Weise erhaltenen Substanz gab folgende Werte:

Präparat I: 0,3584 g Substanz wurden für eine Stickstoffbestimmung nach Kjeldahl verwandt. Sie erforderten zur Neutralisation 38,8 ccm Schwefelsäure (1 ccm = 0,001418 g N); N = 15,35%.

0,2010 g Substanz enthielten 0,0524 g Asche = 26,07%.

0,1905 » » gaben 0,0560 » $Mg_2P_2O_7$; P = 8,02.

Präparat II: 0,1378 g Substanz wurden zu einer Stickstoffbestimmung nach Kjeldahl verwendet. Sie erforderten zur Neutralisation 13 ccm Schwefelsäure (1 ccm = 0,00162 g N); N = 15,30%.

0,1265 g Substanz gaben bei der Verbrennung 0,1632 g CO_2 und 0,0525 g H_2O; C = 35,10%; H = 4,61%.

0,1180 g Substanz gaben 0,0310 g Asche = 26,26%.

0,1670 » » » beim Schmelzen 0,0494 g $Mg_2P_2O_7$; P = 8,27%.

0,1178 » » » bei der Verbrennung 0,1502 g CO_2 und 0,0480 g H_2O; C = 34,78%; H = 4,53%.

Wenn man das Kupfer in Rechnung zieht, war die Zusammensetzung der Säure:

	Präparat I	Präparat II	Älteres Präparat
C	—	37,78%	36,40%
H	—	4,86%	5,10%
N	16,62%	16,51%	17,30%
P	8,99%	8,91%	9,03%
(Basen —	7,7 %	7,35%	11,56%)

Somit differierte die elementare Zusammensetzung der Säure nur wenig von derjenigen, die in meiner früheren Mitteilung angegeben war. Die Verschiedenheit erklärt sich wohl aus der Anwendung von Ammoniak bei der früheren Dar-

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stellung; denn die Erfahrung hat ergeben, daß es beinahe unmöglich ist, aus der Nucleinsäure irgend ein Mineralsalz völlig zu entfernen, das im Laufe der Darstellung angewandt wurde.

Zur hydrolytischen Spaltung wurde das Kupfersalz verwendet. Zur Beurteilung des Purinbasengehaltes wurde die Säure das eine Mal in einer 2%igen, das andre Mal in einer 5%igen Schwefelsäurelösung drei Stunden lang im Autoklaven erhitzt. Da Steudel[1]) in seiner Mitteilung über den Abbau der Thymusnucleinsäure das Vorhandensein von vier Purinbasen im Molekül angibt, bemühte ich mich, besonders Xanthin und Hypoxanthin unter den Spaltungsprodukten der Milznucleinsäure aufzufinden, was aber nicht gelang.

Die Einzelheiten der Analyse lasse ich folgen.

64 g lufttrockenes Kupfersalz, enthaltend 45 g Säure, wurden im Autoklaven bei 125—150° C. mit 400 ccm 2%iger Schwefelsäure zersetzt; darauf wurde filtriert, das Filtrat mit Ammoniak neutralisiert und auf ein sehr kleines Volumen eingedampft. Die dabei entstehende Ausscheidung wurde abfiltriert und enthielt Guanin, sowie etwas Adenin und Phosphorsäure. Sie wurde in verdünnter Schwefelsäure gelöst, die Schwefel- und Phosphorsäure durch überschüssiges Baryumhydrat und dieses wieder durch Schwefelsäure entfernt. Das Filtrat vom Baryumsulfat wurde jetzt eingedampft und mit ammoniakalischer Chlorsilberlösung behandelt. Die Zersetzung des so gebildeten Niederschlages wurde durch Salzsäure bewirkt, worauf das Filtrat vom Chlorsilber mit Ammoniak neutralisiert und auf ein kleines Volumen eingedampft wurde. Nachdem das so ausgeschiedene Guanin abfiltriert war, wurde das Filtrat zu dem des ersten rohen Guaninniederschlages hinzugefügt und mit ammoniakalischer Chlorsilberlösung versetzt, worauf ich den entstandenen gelatinösen Niederschlag mit Salzsäure zersetzte. Das Filtrat vom Chlorsilber wurde nun mit Ammoniak neutralisiert und eingedampft, die vom Guanin abfiltrierte Lösung in der Hitze mit Pikrinsäure behandelt, und das Adeninpikrat abfiltriert. Aus dem Filtrat wurde die Pikrinsäure mit Schwefelsäure und Äther entfernt und dann wurden die übrigbleibenden

[1]) Diese Zeitschrift, Bd. XLII, S. 165 und Bd. XLIII, S. 402.

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Purinbasen mit ammoniakalischer Chlorsilberlösung gefällt; doch bestand dieser Silberniederschlag nur aus Guanin und Adenin. Somit konnte weder Xanthin noch Hypoxanthin in nennenswerter Menge gefunden werden.

Die Ausbeute an Adeninpikrat betrug 4,5 g.

Das Guanin ging durch ein Mißgeschick verloren. In einem zweiten Versuch wurden 40 g lufttrockenes Kupfersalz, enthaltend 31 g freie Nucleinsäure im Autoklaven bei 125—160° C. mit 175 ccm 5%iger Schwefelsäure gespalten. Die Trennung der Basen erfolgte in der gleichen Weise wie vorher und die Ausbeute an Guanin betrug 0,505 g, an Adeninpikrat 2,8 g.

Die Analyse der Pyrimidinbasen erfolgte so, wie es in der vierten Mitteilung beschrieben ist.

30 g lufttrockenes Kupfersalz, enthaltend 21 g freie Säure, wurden verwandt.

Die Ausbeute betrug: Thymin 1,2 g
Cytosinpikrat 4,5 »

Das Uracil wurde nicht gewogen, da die Ausbeute im allgemeinen sehr gering ist.

Berechnet auf 100 g	
Adeninpikrat	8,27 g
Guanin	1,62 »
Thymin	5,71 »
Cytosinpikrat	21,43 »

In einer früheren Mitteilung ist gezeigt worden, daß die Beschaffenheit der Mehrzahl der Nucleinsäurekomponenten wohl bekannt ist, man aber über die Art ihrer Verknüpfung im Molekül der Säure bisher wenig weiß. Neuerdings versuchten Schmiedeberg[1]) und seine Schüler zu zeigen, daß die Nucleinsäure aus einer Grundsubstanz bestehe, die sie Nucleotinphosphorsäure nennen, und die mit den Purinbasen eine verhältnismäßig lockere Bindung zur Bildung des Nucleinsäuremoleküls eingeht. Vorher schon hatten Kossel und Neumann[2]) ein purinfreies Derivat der Nucleinsäure erhalten, welches sie als Thyminsäure bezeichneten. Die Zusammensetzung dieser Thymin-

[1]) Arch. f. exp. Path. u. Pharm., Bd. LXIII, S. 57, 1900.
[2]) Diese Zeitschrift, Bd XXII, S. 77.

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säure ist sehr wenig weiter untersucht worden und die hypothetische Nucleotinphosphorsäure konnte bisher nicht in reiner Form erhalten werden. Doch teilen alle Autoren die Ansicht, daß auf dem Wege der Spaltung in einfache kristallinische Komponenten, intermediär Produkte verschiedener Zusammensetzung gebildet werden, und das Studium solcher intermediärer Produkte scheint von großer Bedeutung für die Erweiterung unserer Kenntnisse betreffs der Bindungsart der einfachen Komponenten im Molekül der Nucleinsäure.

In einer früheren Mitteilung berichtete der Verfasser,[1]) daß es bei der Spaltung mit 2%iger Schwefelsäure gelingt, eine in Wasser und sehr verdünnten Säuren unlösliche Substanz zu erhalten. Diese Substanz enthielt Stickstoff, Phosphor, nur Spuren von Purin- und Pyrimidinbasen, Spuren von Furfurol gebender Substanz und nachweisbare Mengen eines Körpers, der bei weiterer Spaltung mit Mineralsäuren Lävulinsäure liefert. Ob die andern Komponenten aus dem Molekül in freiem Zustande oder miteinander vereinigt abgespalten waren, wurde nicht konstatiert. Im Laufe dieser Untersuchung sollte nun auch der Versuch gemacht werden, außer diesem schon beschriebenen Produkt noch andere komplexe Moleküle unter den durch Mineralsäuren gebildeten Spaltungsprodukten der Nucleinsäure aufzufinden. Dies geschah folgendermaßen:

Nucleinsäure wurde mit Hilfe von verdünnten Säuren in eine lösliche und eine unlösliche Fraktion gespalten. In einem Teil der löslichen Fraktion (I) wurde eine Bestimmung der Pyrimidinbasen vorgenommen, ein anderer Teil (II) wurde eingedampft und mit einer 25%igen Lösung von Schwefelsäure weitergehend gespalten, worauf in dem Reaktionsprodukt ebenfalls die Menge der Pyrimidinbasen festgestellt wurde. Wenn komplexe Moleküle in der Lösung I vorhanden waren, so hätte bei dem zweiten Versuch (in Fraktion II) die Ausbeute an Pyrimidinbasen größer sein müssen. Die Produkte der zweiten Spaltung der löslichen Fraktion wurden dann verglichen mit denen des unlöslichen Restes.

[1]) American Journal of Physiology, Bd. XII, S. 213, 1904.

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Die Verteilung der verschiedenen Körpergruppen war die folgende:

Purinbasen.

Im wesentlichen wurden alle Purinbasen aus dem Molekül durch Erhitzen der Substanz mit 2%iger Schwefelsäure auf 150° entfernt und dies stimmt überein mit der Tatsache, daß bei der weiteren Behandlung mit 5%iger Säure die Ausbeute im wesentlichen unverändert bleibt. Die unlösliche Fraktion enthält nur Spuren dieser Basen.

Pyrimidinbasen.

Versuch I. 40 g lufttrockene Substanz, enthaltend 5,29% Phosphor, wurden im Autoklaven mit 100 ccm 25%iger Schwefelsäure vier Stunden auf 150—175° C. erhitzt. Unter den hydrolytischen Produkten fand sich Thymin und Cytosin in folgender Ausbeute:

Thymin	1,2 g
Cytosinpikrat	3,1 »

Versuch II. 40 g der gleichen Substanz wurden in 250 ccm 5%iger Schwefelsäure im Autoklaven bei 125—150° C. vier Stunden lang erhitzt. Unter den Produkten der Hydrolyse wurden die Pyrimidinbasen nach der Methode von Kossel-Jones bestimmt und gaben folgende Ausbeute:

Thymin	0,45 g
Cytosinpikrat	1,2 »

Versuch III. 40 g derselben Substanz wurden in der gleichen Weise wie im letzten Versuch gespalten. Die lösliche Fraktion wurde auf 100 ccm eingedampft und soviel konzentrierte Schwefelsäure hinzugefügt, daß ein Säuregehalt von 25% erzielt war. Darauf wurde die Lösung vier Stunden im Autoklaven erhitzt. Die Ausbeute an Pyrimidinbasen war

Thymin	1,4 g
Cytosinpikrat	2,7 »

Versuch IV. Die bei den Versuchen II und III durch Hydrolyse mit 5%iger Schwefelsäure erhaltenen unlöslichen Rückstände wurden vereinigt und mit 25%iger Schwefelsäure

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gespalten; es ergab sich kein Thymin und nur eine Spur von Basen, die ein unlösliches Pikrat bildeten.

Aus diesen Versuchen ergibt sich, daß bei der Hydrolyse der Milznucleinsäure mit 5%iger Schwefelsäure die folgenden Reaktionen stattfinden: Praktisch werden alle Purinbasen in Freiheit gesetzt, desgleichen ein Teil der Pyrimidinbasen, während der Rest in Form eines Molekülkomplexes verloren geht. (Die Natur dieser Substanz soll in einer besonderen Mitteilung erörtert werden.) Es entsteht ein unlöslicher Körper, der frei von Purin- und Pyrimidinbasen ist.

Kohlehydrate.

In einer früheren Mitteilung war festgestellt worden, daß bei der Hydrolyse mit 2%iger Schwefelsäure ein Rückstand entsteht, der nicht mehr die Furfurol gebende Substanz enthält, jedoch bei weiterer Spaltung noch Lävulinsäure liefert. Bei der vorliegenden Untersuchung sollte nun versucht werden, festzustellen, wie die Hexose in den verschiedenen Fraktionen verteilt ist, die man bei der Hydrolyse der Milznucleinsäure mit Hilfe von verdünnten Mineralsäuren erhält, zu welchem Zwecke folgende Versuche ausgeführt wurden:

Versuch I. 10 g der auch in den früheren Versuchsreihen angewandten Substanz wurden mit 50 ccm 25%iger Schwefelsäure in einem Autoklaven bei 150° C. drei Stunden lang erhitzt. Aus den Produkten der Hydrolyse wurde dann das Silbersalz der Lävulinsäure auf die gewöhnliche Weise erhalten. Ausbeute: 0,7 g.

Versuch II. 40 g der gleichen Substanz wurden im Autoklaven bei 150° C. vier Stunden lang mit einer 5%igen Schwefelsäurelösung hydrolysiert, darauf wurde filtriert und der Rückstand mit Wasser ausgewaschen. Nach dem Eindampfen der vereinigten Filtrate und Waschwässer auf 100 ccm wurden 15 ccm konzentrierte Schwefelsäure hinzugefügt und die Lösung vier Stunden im Autoklaven bei 150° C. erhitzt. Das Silbersalz der Lävulinsäure, welches in der gewöhnlichen Weise gewonnen war, wog 3,4 g.

Versuch III. 4 g des nach der Spaltung mit 5%iger

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Schwefelsäure erhaltenen, getrockneten Rückstandes wurden im Autoklaven mit 25%iger Schwefelsäure gespalten, worauf sich nur eine Spur Lävulinsäure erhalten ließ.

Versuch IV. 20 g des nach Spaltung mit 2%iger Schwefelsäure erhaltenen und getrockneten Rückstandes wurden im Autoklaven mit 25%iger Schwefelsäure gespalten und es ergaben sich nur ungefähr 0,150 g Thymin und 0,750 g Cytosinpikrat. Die Ausbeute an Silbersalz der Lävulinsäure betrug 0,5 g.

Aus diesen Versuchen geht hervor, daß bei der Hydrolyse mit 5%iger Schwefelsäure alle Kohlehydrate des Moleküls der Nucleinsäure sich in den löslichen Spaltungsprodukten befinden. Ob sie in freiem Zustande oder in Form eines Molekülkomplexes vorhanden sind, soll später untersucht werden. Bei der Spaltung mit 2%iger Schwefelsäure entsteht eine unlösliche Substanz, in welcher das Verhältnis von Kohlehydraten zu Pyrimidinbasen größer als in der ursprünglichen Säure ist, obgleich die absolute Ausbeute an Lävulinsäure niedriger ist, als die bei der Hydrolyse der Nucleinsäure erhaltene.

Melanin.

Bei der Hydrolyse von Nucleinsäuren mit starken Mineralsäuren erhält man Substanzen, die wohl als Melanine zu betrachten sind. Dieser Substanz haben Schmiedeberg, Osborne und Harris ihre Aufmerksamkeit zugewandt.[1]) Die Substanz des ersten Untersuchers enthielt Phosphor, die von Osborne und Harris beschriebene war frei davon. Den Ursprung der Substanz dachte man sich in der Weise, daß sie durch Kondensation aus den Spaltungsprodukten von Purinbasen und Kohlehydraten entstanden sei.

Im Laufe der gegenwärtigen Untersuchung ergaben sich folgende Beobachtungen:

Versuch I. 40 g der bei der ersten und zweiten Versuchsreihe verwendeten Substanz wurden mit 5%iger Schwefelsäure gespalten. Der lösliche Teil wurde nun eingedampft und mit 25%iger Schwefelsäure weiter erhitzt. (Diese Fraktion

[1]) Diese Zeitschrift, Bd. XXXVI, S. 83, 1902.

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enthielt, wie aus der ersten und zweiten Versuchsreihe zu entnehmen ist, alle Kohlehydrate, sowie die Purin- und Pyrimidinderivate des Nucleinsäuremoleküls.) Die Ausbeute an Melanin war in dieser Fraktion sehr unbedeutend.

Versuch II. 4 g des getrockneten Rückstandes, der bei der Hydrolyse mit 5%iger Schwefelsäure erhalten war, wurde der weiteren Zersetzung und zwar mit 25%iger Schwefelsäure unterworfen. Der Erfolg war die Bildung von 1,8 g Melanin, frei von Phosphor und unlöslich in Alkalien und Säuren.

Aus diesen Versuchen ergibt sich, daß Melanin und sein phosphorhaltiger Vorläufer entweder aus denjenigen Bestandteilen der Nucleinsäure entstehen, die weder Purinbasen noch Kohlehydrate sind, oder aber, daß sie durch Kondensation aus den Spaltungsprodukten der Kohlehydrate und Purinbasen gebildet werden. In letzterem Falle müßte die Kondensation einfach beim Erhitzen mit 5%iger Schwefelsäure stattfinden.

Um mehr Klarheit über den Prozeß der Melaninbildung zu bekommen, schien es ratsam, die Zusammensetzung der beiden Vorstufen — nämlich des Rückstandes nach Hydrolyse mit 2%iger Schwefelsäure und des anderen nach Spaltung mit 5%iger Schwefelsäure — mit der des Melanins und der der ursprünglichen Nucleinsäure zu vergleichen. Es wurde also die Aufmerksamkeit den Spaltprodukten der beiden Mutter-substanzen des Melanins zugewandt. Sie sowohl wie das Melanin enthielten Kupfer und somit schien es zweckmäßig, die Vergleiche mit dem Kupfersalz der Nucleinsäure anzustellen.

Die Resultate der Analyse waren folgende:

I. Rückstand nach Hydrolyse mit 2%iger Schwefelsäure:

0,2220 g Substanz wurden zu einer Stickstoffbestimmung nach Kjeldahl verwendet; sie erforderten 29,6 ccm Schwefelsäure zur Neutralisation (1 ccm Säure = 0,00124 g N); N = 16,53%.

0,3380 g Substanz wurden zu einer Phosphorbestimmung verwendet. Sie gaben 0,0224 g $Mg_2P_2O_7$; P = 1,85%.

0,2352 g Substanz gaben 0,0682 g Asche = 29%.

0,2396 » » » bei der Kupferbestimmung 0,0690 g CuO; Cu = 21,75%.

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II. Rückstand nach Hydrolyse mit 5%iger Schwefelsäure:

0,2683 g Substanz wurden zu einer Stickstoffbestimmung nach Kjeldahl verwendet. Sie erforderten 48,2 ccm der Schwefelsäure des vorigen Versuches. N = 22,29%.

0,2099 g Substanz lieferten 0,666 g Asche = 31,25%.

0,2863 g Substanz wurden zu einer Phosphorbestimmung verwendet. Die Ausbeute war sehr gering.

0,1925 g Substanz gaben bei der Kupferbestimmung 0,0640 g CuO; Cu = 19,05%.

III. Melanin:

0,2730 g Substanz erforderten bei einer Stickstoffbestimmung nach Kjeldahl 22,6 ccm der Schwefelsäure der vorigen Versuche. N = 10,28%.

0,2894 g Substanz lieferten 0,0590 g Asche = 20,39%

0,2200 g Substanz lieferten bei der Phosphorbestimmung keine Ausbeute.

0,2700 g Substanz lieferten bei der Kupferbestimmung 0,0536 g Kupferoxyd. Cu = 15,87%.

In der folgenden Tabelle sind zum Vergleiche die Analysenresultate der vorherigen Substanzen und die der Nucleinsäure zusammengestellt; daneben findet sich die Zusammensetzung einer Substanz, die durch Hydrolyse einer freien Nucleinsäure (nicht des Kupfersalzes) mit 2%iger Schwefelsäure entsteht. Sie ist in einer früheren Mitteilung beschrieben.

	Nucleinsäure	Rückstand I (Cu-Salz)	Rückstand I (freie Säure)	Rückstand II	Melanin
N	15,35%	16,53%	8,83%	22,29%	10,28%
P	8,10%	1,85%	8,62%	wenig	0
Cu	—	21,75%	—	19,05%	15,87%
Asche	26,0%	29,0%	39,0%	31,25%	20,39%

Somit findet bei der hydrolytischen Bildung der Muttersubstanz des Melanins aus der Nucleinsäure zuerst eine Steigerung des Stickstoffgehaltes statt. Dem folgt ein Absinken des Stickstoffes im Melanin; auch ist eine allmähliche Abspaltung von Phosphor zu verzeichnen. Der Kupfergehalt zeigt eine Erhöhung in den Muttersubstanzen des Melanins, weniger im Melanin selbst.

Um über die Natur der Spaltungsprodukte der Vorstufen des Melanins näheres zu erfahren, werden folgende Versuche angestellt:

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20 g trockene Substanz vom Rückstand I wurden mit 25%iger Schwefelsäure im Autoklaven bei 150—175° C. gespalten. In einem Teil des Filtrates wurde die Stickstoffmenge bestimmt und der Stickstoff der nichtbasischen Substanzen aus dem des Filtrates einer Phosphorwolframsäurefällung berechnet. Die Bestimmung des Ammoniaks geschah nach einer von Dr. Stookey und dem Verfasser[1]) angegebenen Methode.

4 g der getrockneten Substanz vom Rückstand II wurden in der gleichen Weise behandelt.

Die Verteilung des Stickstoffes ergab sich wie folgt:

	Rückstand I	Rückstand II
Gesamt-N	1,30 g	0,445 g
Ammoniak-N	13,5% des Gesamten	23,6% des Gesamten
Basisches N	40,5%	65,8%
Nichtbasisches N	45,5%	10,68%

Es wurde schon erwähnt, daß der Rückstand I bei der Hydrolyse Lävulinsäure lieferte, ferner etwas Thymin und eine Base, die ein unlösliches Pikrat bildete.

Bei der Spaltung des Rückstandes II konnte weder Lävulinsäure noch Thymin erhalten werden und nur 0,250 g eines unlöslichen Pikrates.

Es ist bemerkenswert, daß bei der Hydrolyse der freien Nucleinsäure (anstatt des Kupfersalzes) mit 2%iger Schwefelsäure[2]) eine Substanz mit 11,33% Phosphor erhalten wird, während das Kupfersalz unter den gleichen Bedingungen einen Körper mit 2,45% Phosphor und mit 21,75% Kupfer liefert. Das weist auf die Tatsache hin, daß jene Kondensation schon stattgefunden hat. Auch ist es bemerkenswert, daß aus dem Melanin das Kupfer weder mit Ammoniak noch mit Salzsäure entfernt werden kann, somit also in einer noch stabileren Form als in der eines Salzes vorhanden zu sein scheint.

[1]) Journal of Medical Research, Bd. V (neue Folge), S. 455, 1903.
[2]) American Journal of Physiology, Bd. XII, S. 213, 1904.

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Reprinted from The Journal of Experimental Medicine, 1905.

ON THE RATE OF ABSORPTION FROM INTRAMUSCULAR TISSUE.

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(*From the Rockefeller Institute for Medical Research.*)

Plates I.–III.

The rate of absorption from muscles has never yet been a subject of scientific investigation. In fact, nowhere in physiological literature do we meet the suggestion that absorption from the muscles might differ from that of any other parenchymatous or subcutaneous tissue. Only from the serous cavities, especially the peritoneal, is it generally assumed that absorption takes place more rapidly than from the subcutaneous tissues. In the recent extensive studies of infections and immunity, in which inoculation of animals with virulent, toxic, or antitoxic material is one of the most extensively employed methods of investigation, this is usually accomplished by subcutaneous or intraperitoneal injections, to which injections into the spinal canal, the brain, or the nerve trunks were recently added. In the latter cases, the injections are carried out rather to demonstrate their localized effect than to cause a general systemic infection or intoxication. Intramuscular injection, however, as far as we know, was never yet employed, at least not consciously, as a method of introducing infectious or toxic products into the circulation.

In therapeutics, however, we now and then come across the practice of intramuscular administration of drugs. It originated in the treatment of syphilis with mercury. When at the beginning of the '80's of the last century it was found that hypodermic injections of any compound of mercury gave considerable pain and favored the development of abscesses at the site of injection, the method developed of giving deep-seated injections,

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especially in the gluteal muscles where it was claimed that they caused neither pain nor abscesses. It was, however, especially stated by certain authorities[1] "that the absorption from the intramuscular tissue with its fewer lymphatics is much slower than from the subcutaneous tissues where the lymphatics are abundant." For the same reason, namely, of avoiding pain and abscess, the practice of injecting into the gluteal muscles extended to some other drugs, such as digitalis, camphor, etc., which cause pain when injected subcutaneously.

OUR EXPERIMENTS.

We came across the question of the rate of absorption from the intramuscular tissues while studying the effect of suprarenal extract upon the pupil when injected peripheral to a ligature applied to an extremity. In studies of the effect of suprarenal extract upon the paradoxical pupil dilatation,[2] the authors stated that in animals from which one superior cervical ganglion was previously removed, a subcutaneous injection of adrenalin caused a long and lasting dilatation of the pupil on the side without ganglion. The dilatation set in about fifteen minutes after the injection. In order to demonstrate that the suprarenal extract is not oxidized by the tissues, the authors ligated a leg and injected adrenalin peripheral to the ligature. When the ligature was removed after some time, the pupil on the side without ganglion dilated. In this case, however, the dilatation appeared a minute or two after removal of the ligature. The problem which the present writers set out to study was the cause of this rapid onset of the effect of the extract after the use of the ligature. We soon made the observation that in introducing the extract into the part of the extremity peripheral to the ligature, the injections were almost unavoidably made intramuscularly. We then began to study directly the rate of absorption after intramuscular injections, and we reached the remarkable result that, for the substances we have studied so far, the effect of an intra-

[1] Neumann und Finger, *Wiener Medizin. Presse*, 1885, xxvi., 80.

[2] S. J. Meltzer and Clara Meltzer Auer, *American Journal of Physiology*, 1904 xi, 28.

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muscular injection stands nearest to that of an injection into the circulation, and is far above that of a subcutaneous application.

The substances we have studied so far are: adrenalin, curare, fluorescein, and morphine. In order to obtain clear results it was desirable to employ substances which give distinct reactions, and at the same time permit a comparison of the different modes of application in the same animal. The most available substance for our purpose was again suprarenal extract, as its absorption into the blood is capable of giving us three distinct reactions: the rise of blood-pressure, the dilatation of the pupil, and the characteristic prostration. The effect upon the blood-pressure has, as a method of investigation, an advantage over the other reactions mentioned in that as it permits a graphic presentation of the results.

THE EFFECT OF INTRAMUSCULAR INJECTIONS OF ADRENALIN UPON THE BLOOD-PRESSURE.

The blood-pressure experiments, as well as all other experiments recorded in this paper, were made exclusively on rabbits.

In the experiments on blood-pressure the carotid artery was connected with a mercury manometer, by means of which the variations in the pressure were recorded on a slowly revolving smoked drum. During the operations the animals were anæsthetized with ether; during the further course of the experiments, in some cases the animals were kept under mild anæsthesia by ether, or a small dose of morphine was injected. In a few cases curare was given intramuscularly.

It is worth while mentioning that, in studying the effect of suprarenal extract upon the blood-pressure in rabbits, we were greatly hampered by the favoring effect of the extract upon blood-clotting.[3] As soon as the extract was absorbed into the circulation we could hardly obtain two consecutive tracings without being compelled to free the cannula and connecting tubing from clots. In some cases a clot ended the tracing before the entire effect of the extract was over. In other cases the

[3] Vosburgh and Richards, *American Journal of Physiology*, 1903, xi, 30.

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cleaning of the tube was of no avail, as the clot was formed far down in the carotid artery.

On the other hand, a *saturated* solution of magnesium sulphate, with which cannula and connecting tube were filled, proved to be a source of great danger to the animal. When, after a struggle, the pressure sank for a few seconds, the solution of magnesium sulphate rushed backward through the central end of the carotid and reached the aorta, the animal was liable to die within a few seconds without any asphyxia and with practically no struggle. We preferred using less concentrated solutions of magnesium sulphate, and contending with the difficulties of clotting rather than risk the effect upon the circulation. We lost a number of animals in rapid succession, due to this effect of the magnesium salt. We bring out this point here because we believe that some physiological experimenters are not fully familiar with the eminently toxic effect of magnesium sulphate when brought directly into the circulation.

In studying the effects of intramuscular injections of suprarenal extract upon the circulation, we had to compare them with the effects of other modes of administration, i. e., with the effects of subcutaneous, intraperitoneal, and intravenous injections of the extract. This comparison extended to the questions of the time of the onset, of the effect after injection, the character of the development, and the degree of the increase of the pressure which is caused by each mode of injection. For various reasons we had to neglect in this study the question as to the duration of the effect due to each mode of injection.

Of the modes of administration of the extract, we have compared the intramuscular most extensively with the subcutaneous injections from which they are generally but little distinguished. We made but few comparisons with intravenous injections, considering this comparison of no particular importance in the present study; and we also made but few comparisons with intraperitoneal injections, for reasons which we shall state later. Of the results which were obtained, the greatest value had to be attached to those which were derived from comparisons made on one and the same animal, as in comparisons between the

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results obtained from different individuals the element of individuality might come into play. But in the comparisons on the same animal the question of sequence had to be taken into consideration, since a preceding injection always has a better effect than the following one.[4] We have therefore, in some experiments, varied the sequence of injections, i. e., in some experiments a subcutaneous injection was given first and this was followed by an intramuscular one; and in others we reversed the order. However, since a preceding intramuscular injection impairs incomparably more the effect of a subsequent subcutaneous injection than a subcutaneous could effect a following intramuscular one, we began the majority of experiments with a subcutaneous injection.

Between two injections we usually permitted intervals of fifteen to thirty minutes to elapse, sometimes letting the drum run continuously or obtaining short curves every two minutes. Although in most of the experiments we waited with the second

[4] It has been stated by many observers that in consecutive intravenous injections of suprarenal extract the effect upon the blood-pressure is gradually diminished. This gradual decrease in the effect was looked upon as a sort of process of adaptation or immunization. In previous unpublished experiments with subcutaneous injections of the extract we have noticed that by this mode of administration we rarely obtained an effect from a second and certainly not from a third injection, although they were given each time in another place and the effects of subsequent intravenous injections were in no way impaired. The above given explanation of adaptation, etc., will not hold good in this case. In our present studies we also found that in repeated intramuscular and peritoneal injections of adrenalin the effect upon the blood-pressure is rapidly decreased. Our interpretation of this phenomenon is as follows: In a previous paper (Meltzer and Auer, *Transactions of the Association of American Physicians*, 1904, xix, 207) we have demonstrated that the introduction of suprarenal extract into the circulation retards the absorption from the tissues into the blood, and also the transudation from the blood into the tissues. When, therefore, a subcutaneous, an intraperitoneal, or an intramuscular injection of suprarenal extract once produces an effect upon the blood-pressure, it has then of course reached the circulation and causes therefore a retardation of the further absorption of the extract from the tissues; hence the smaller effect of a second injection. Possibly the same explanation might also hold good for the gradual reduction of the effect of repeated intravenous injections of the extract, since transudation also becomes retarded, and, since even in intravenous injections, the vascular intima has to be permeated before the extract can exert its influence upon the muscle fibres of the media.

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injection until the blood-pressure reached its original level or fell below it, we cannot claim that even in those cases the effect of the preceding injections had actually passed off, since the frequent cleanings of the cannula and the necessity of letting blood escape in order to drive out a deep-seated clot, might have interfered with the absolute pressure. For this reason we refrained, as stated above, from utilizing the data regarding the duration of the effects of these injections.

We did not attempt to make an exhaustive study of the effects of subcutaneous and intraperitoneal injections of the suprarenal extract upon the blood-pressure, since the chief purpose of our present investigation was a study of intramuscular absorption, and not of the effect of suprarenal extract upon blood-pressure. However, we have in the present and in previous studies acquired a few data regarding the effects upon blood-pressure of subcutaneous, as well as of intraperitoneal, injections of suprarenal extract which we are about to report briefly.

SUBCUTANEOUS INJECTIONS.

In the physiological and the pharmacological literature upon suprarenal extract we frequently find the brief statement that subcutaneous injections of the extract exert no influence upon blood-pressure. In the medical literature, however, some scattered remarks are met that subcutaneous injections do increase the blood-pressure. We are not aware that any definite data on the subject accompanied by tracings or figures have been published anywhere. From our present studies we know that if special care is not taken, the hypodermic needle will sometimes get under the fascia of muscles and the result will be a definite effect upon the blood-pressure, but it is then not from a subcutaneous, but from an intramuscular injection of the extract. In the light of our present experience we have reason to distrust therefore not only the statements of others, but also our own previous observations when made while we had no idea of the necessity of sharply distinguishing between subcutaneous and intramuscular injections. However, as will presently be shown,

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there is something characteristic about the blood-pressure curve obtained from a subcutaneous injection which assists in identifying it.

From our experiments we may state in a general way that subcutaneous injections of suprarenal extract in rabbits will undoubtedly influence the blood-pressure, but the degree of the effect is in no way comparable to that obtained by intravenous injections. Furthermore, the effect is very variable; while sometimes even a comparatively small dose will bring out a relatively powerful effect, a large dose cannot always be relied upon to cause a noteworthy rise of pressure. As a rule, a dose of 1.5 cc. of commercial adrenalin (1:1000) will cause the characteristic prostration of the animal and the ears will show an absolute blanching, while at the same time the general blood-pressure may show a gradual rise not exceeding perhaps ten millimetres of mercury. On the other hand, doses of 0.4 or 0.5 cc. of adrenalin per kilo animal will, as a rule, exert no influence whatsoever upon the blood-pressure of the rabbit. (See Figs. 4, and 6.) But in some exceptional cases even such a small dose might unexpectedly produce a comparatively powerful influence. Figure 1, presents such a powerful effect of a first subcutaneous injection of 0.8 cc. adrenalin in a rabbit weighing 2200 grams. In a series of experiments on more than thirty rabbits such effects were obtained only twice.

Figure 1 might serve also as prototype for the character of the normal curves, which are obtained after a subcutaneous injection, no matter whether the effect be small or large. While the rise of pressure after intravenous injections is characterized by a very short latent period, steep rise and gradual descent, the entire effect lasting only from four to six minutes, we see in subcutaneous injections the effect only beginning minutes after the injection, the pressure rising slowly, remaining at the maximum for some time, and then gradually descending to the original level, the entire course frequently extending over a period of twenty to thirty minutes.

The original height of the blood-pressure seems to be an influential factor upon the effect of a subcutaneous injection. When

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the blood-pressure is low we may reasonably expect a noteworthy rise after such an injection. However, a rise of more than 25 to 30 millimetres of mercury is under all conditions rather an exception.

We have already stated above that, as a rule, only the first injection is distinctly effective; the effect of a second injection, if there is any, is already perceptibly reduced, and there is rarely any effect after a third injection. However, after an interval of a few hours an injection might again become effective.

A struggle of the animal seems to have a tendency to bring out the effect of a subcutaneous injection. In normal conditions, struggle, as a rule, causes a fall of short duration, and if there is sometimes a moderate rise of pressure, it returns after a few seconds to the previous level. After a subcutaneous injection of an ineffectual dose of adrenalin we noticed, however, in a few instances that a struggle was followed by a sudden moderate but long-lasting rise of pressure (see Fig. 10.) Possibly struggle is instrumental in causing rapid absorption of some of the injected adrenalin.

INTRAPERITONEAL INJECTIONS.

Our experience with the effects of intraperitoneal injections of suprarenal extract were a surprise and a puzzle to us. In the majority of the experiments we have seen either no effect or a rise of only 10 or 20 millimetres. (See Figs. 13 and 14.) We met only with two exceptions; but in these two instances the rise which followed immediately after the injections was so powerful and so very like the effect of an intravenous injection, as to raise the doubt whether the injections were not indeed made intravenously. At first we made the injections by means of a hypodermic syringe through the intact abdominal wall. We soon discarded this method and carried on the injections either through a small nick in the abdominal wall or through wide incisions, trying at the same time to carry the adrenalin to different parts of the peritoneum, with the above stated results. Of the two exceptions mentioned, one was obtained by plunging a hypodermic syringe through the intact wall,

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the other by pushing a hard catheter through a small abdominal incision, between liver and diaphragm. In the first instance the needle might have entered a blood-vessel, and in the second the catheter might have injured the surface of the liver, thus facilitating the entrance of the extract directly into the circulation.

How are these results to be explained? Can it indeed be a fact that suprarenal extract enters but little into the circulation when applied directly to the peritoneum, possibly on account of its intense local action on the blood-vessels of the peritoneum, thus preventing its absorption into the blood? We intend to take up this question again in an investigation specially devoted to it, and shall therefore refrain from discussing it here any further.

INTRAMUSCULAR INJECTIONS.

Our experience with intramuscular injections of suprarenal extract has been more extensive and more varied than with the other methods. The results, however, were so precise and uniform that they can be stated briefly. The effect of an intramuscular injection of adrenalin upon the blood-pressure approaches in every respect that caused by an intravenous injection. The rise sets in a few seconds after the injection and reaches within a fraction of a minute its maximum, which can be considerable. A dose of 0.3 or 0.4 cc. of adrenalin may produce a rise of 40 to 50 millimetres of mercury. Figures 5, 9, 15, will illustrate these effects better than they can be described by words. These curves were obtained from intramuscular injections of such small doses of adrenalin that if applied subcutaneously they exerted an insignificant influence or none at all upon the blood-pressure. The effect of an intramuscular injection differed from that of an intravenous only in degree; the rise of pressure was not as large and not entirely as sudden as after intravenous injections. Furthermore, an intravenous injection still responded with a fairly good rise when, after repeated intramuscular injections, no reaction could any longer be obtained. We have, however, to state that in intramuscular injections the response was not exhausted until three or more consecutive

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injections had been made, although each consecutive reaction became distinctly smaller.

A feature of our intramuscular curves is the frequent appearance of so-called "vagus-pulse," i. e., the heart-beats become larger and often less frequent, as was ascribed by Biedl and Reiner [4] (in tracings from intravenous injections) to a stimulation of the vagus centre in the medulla. From a comparison of the blood-pressure tracings in the experiments of many writers on intravenous injections with the tracings obtained by us after intramuscular injections, it would appear to us that the vagus-pulse is a much more frequent phenomenon after an intramuscular administration of suprarenal extract than after an intravenous one. We record here our observations without further comment as to the possible nature of this difference. Sometimes the vagus-pulse sets in before the pressure has reached its maximum; then the pressure will rise still higher after the cessation of the vagus-pulse (see Figs. 5 and 14); or the large pulsation sets in after the rise has attained its maximum, and it will then be accompanied by a slight fall of pressure (see Fig. 15), or there will be large vagus-pulses throughout the entire effect following the injection, the rise of pressure in this case not being very high (see Fig. 7).

In some instances it seemed that also after an intramuscular injection a struggle would increase the rise of blood-pressure, or cause a second rise after there was already a tendency to drop.

As to the duration of the rise we cannot, for reasons mentioned, make any positive statements. We may, however, remark that the general impression received from a survey of our tracings is that the effect upon the blood-pressure lasts somewhat longer after intramuscular injections than after intravenous ones.

In the majority of the experiments the injections were made into the muscles of the back, in the lumbar region, and we had not a single failure. We have, however, established that the effect is the same if the injections are made in any other muscle.[5]

[4] *Pflüger's Archiv*, 1898, lxxviii., 385.

[5] Among the tracings in our possession from previous studies on the effects

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In injecting into other muscles of the rabbit's body care has to be taken that the needle remains within the muscle, as with the thin muscles of the animal it might easily happen that the needle enters the loose areolar tissue between groups of muscles, in which case the effect will be the same as from the subcutaneous tissue.

EXPERIMENTS WITH THE PARADOXICAL PUPIL REACTION.

As was already stated above, S. J. Meltzer and Clara Meltzer Auer [6] have found that some time after removal of the superior cervical ganglion (twenty-four hours in rabbits and forty-eight hours in cats) a subcutaneous injection of adrenalin caused a dilatation of the pupil on the side without the ganglion, which would last for hours. The doses of adrenalin employed were about 1.2 to 1.5 cc. for a "large rabbit," and the reaction appeared "a few minutes" after the injection. In some instances even such a small dose as 0.6 cc. caused some dilatation. The authors had at that time, of course, no reason for distinguishing between subcutaneous and intramuscular injections.

We now resected the superior cervical ganglion in a number of animals and studied the effect of the extract according to the tissue injected, with the following results:

When a dose of adrenalin of 0.4 cc. per kilogram of animal was injected intramuscularly, the pupil on the operated side began to dilate within a fraction of a minute and reached the maximum within two minutes. Such a small dose has nearly no effect upon the pupil in subcutaneous application. When a large dose was injected subcutaneously, the pupil on the operated side dilated ten to fifteen minutes after the injection. When the pupil once became dilated *ad maximum*, the dilatation lasted apparently just as long after intramuscular injection as after an effective subcutaneous one. Doses smaller than 0.4 cc. per kilo

of subcutaneous injections of suprarenal extract there was one from a "subcutaneous" injection of a small dose of adrenalin made into the pectoral muscle (Fig. 2). It looked exactly like the following tracing (Fig. 3), which was obtained with a still smaller dose from an intravenous injection. We know now that it was not due to a subcutaneous but to an intramuscular injection.

[6] S. J. Meltzer and Clara Meltzer Auer, *Amer. Journ. of Phys.*, 1904, xi, 28.

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given intramuscularly would often cause only a moderate dilatation of the pupil, which would set in later and disappear sooner. In no case have we observed that an effective intramuscular injection has ever caused any dilatation of the pupil on the normal side. This fact distinguishes the intramuscular application from the intravenous, after which even the pupil on the normal side becomes dilated for a fraction of a minute.

THE EFFECT OF THE SUPRARENAL EXTRACT UPON THE SKELETAL MUSCLES.

The early writers on the influence of suprarenal extract have noticed its general prostrating effect upon the animal. Oliver and Schaefer [7] have observed that ten minutes after a subcutaneous injection of the extract into a frog "its movements have usually become languid," and after another 5 minutes" its voluntary power over the muscle is greatly diminished." In the rabbit, "half-an-hour after a large subcutaneous dose, the animal may become listless, and may, when interfered with, move languidly." In our experience a dose of about 2 cc. of adrenalin, when given to a medium-sized rabbit subcutaneously, would produce a distinctly prostrating effect. The animal would lie on its belly, all four extremities stretched out, the chin resting on the table and the head turned sideways by its own weight, and would move only with great effort. This effect appeared fifteen to twenty minutes after the injection. After intramuscular administration, however, the prostrating effect already appeared a minute or two after the injection of 0.6 or 0.8 cc. per kilo, a dose which exerts hardly any effect in subcutaneous injections. We have to add that doses larger than 0.8 cc. can be fatal to the rabbit when given intramuscularly; the animal may die even within a few minutes after the injection, while we have never met with such accidents in the practice of subcutaneous injections even after larger doses.

EXPERIMENTS WITH CURARE.

The experiments with curare were very instructive. They brought out the difference between subcutaneous and intramus-

[7] Oliver and Schaefer, *Journal of Physiology*, 1895, xviii, 227.

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cular injections in a striking manner. We succeeded in finding the doses which in subcutaneous injections brought out no perceptible effect, while after intramuscular injection all the voluntary muscles were paralyzed, at least all except the respiratory muscles. We shall illustrate with a few protocols.

Experiment I. Nov. 15. 1904.—1. Gray male rabbit, 1300 grams injected subcutaneously with 0.25 cc. of a 1 % solution of curare (0.2 cc. per kilo). Rabbit quiet at first. Practically no effect.

2. White and gray male rabbit, 1560, injected intramuscularly, (right thigh) 0.3 cc. of above solution of curare (0.2 cc. per kilo). (The same solution was used in all experiments.) After seven minutes the paralysis developed to such degree that artificial respiration was necessary. Animal used for a blood-pressure experiment.

Experiment II. Nov. 16.—1. Rabbit number 1 of Experiment I was again given subcutaneously 0.25 cc. of curare (0.2 cc. per kilo). Practically no effect.

2. Black and white male rabbit, 1320, received intramuscularly in right thigh, 0.25 cc. curare (0.2 cc. per kilo); paralyzed; artificial respiration necessary. Used for a blood-pressure experiment.

Experiment III. Nov. 17.—Rabbit number 1 of the two previous experiments, during which it had twice received subcutaneously 0.2 cc. curare per kilo with no effect, was now given intramuscularly (thigh) 0.25 cc. of curare (0.2 cc. per kilo). After six minutes practically paralyzed.

Experiment IV. Nov. 30.—1. Brown female rabbit, 1540, injected intramuscularly (back) with 0.29 cc. of the 1 % solution of curare (0.19 cc. per kilo). After five minutes animal unable to move, all voluntary muscles profoundly affected except the muscles of the respiratory mechanism. Two hours later still paretic, but able to move again.

2. Brown female rabbit, 1435, received subcutaneously (back) 0.27 cc. of the solution of curare (0.19 cc. per kilo). Watched for two hours; no effect whatsoever.

The effect of an intramuscular injection of curare resembles

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greatly that of an intravenous one. The effect sets in quite rapidly and lasts for some time, perhaps even longer than after an intravenous injection. We have employed doses so small as would hardly be sufficient to ensure absolute rest even in intravenous injections. When at the end of a blood-pressure experiment, for which some of the rabbits with intramuscular injections of curare were utilized, the animal was permitted to die, the asphyxia would bring out perfunctory convulsions. Even during the experiment one or the other animal would show once in a while a faint struggle, especially when the dose was only 0.18 cc. per kilo; and we have never added any more curare during the experiment to ensure absolute rest, as is frequently done in intravenous injections. We believe that the intramuscular injections of curare will prove to be a practical method in experimentation on animals, as it is much more convenient and less dangerous than intravenous injection.

EXPERIMENTS WITH FLUORESCEIN.

The effects of the subcutaneous injection of fluorescein we have extensively described in our experiments upon the influence of suprarenal extract upon absorption and transudation. The skin and mucous membranes become gradually more or less distinctly yellow. Following larger doses, Ehrlich's line in the pupil appears after some time. By intravenous injection the animal is stained intensely yellow, almost in an instant. We have now tried an intramuscular injection of fluorescein, comparing it with the effect of a subcutaneous administration of a similar dose. We shall quote a protocol of such an experiment.

Nov. 16.—White and gray rabbit, 1050 grams received subcutaneously (back) at 4.05 P.M., 2 cc. of a 5 % solution of fluorescein. At 4.09 2.5 cc. of the same solution of fluorescein was injected intramuscularly (back) into a gray rabbit, 1300 grams (both animals received 1.5 cc. per kilo). At 4.12 conjunctivæ of both animals show yellow tint, but much more intense in the "intramuscular." 4.13, "intramuscular" shows yellow nose, "subcutaneous" still pink. 4.20, "intramuscular" much more

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yellow in all parts than "subcutaneous." Ehrlich's line appears at 4.20 in the left, and at 4.25 in the right eye of the "intramuscular"; no Ehrlich line in either eye of the "subcutaneous." 4.29, both pupils of "intramuscular" perfectly green; the pupils of "subcutaneous" black, no tint of green.

Fluorescein is absorbed incomparably more rapidly and in larger doses from the intramuscular than from the subcutaneous tissue. However, it should also be noted that the effect of an intramuscular injection of fluorescein cannot be compared in rapidity with that of an intravenous injection of the same dose and strength.

EXPERIMENTS WITH MORPHINE.

We have finally to record the results of our experiments made with morphine. They are fully in accord with the results obtained with the injection of the other substances recounted in the preceding pages. The symptoms brought on by morphine are inappropriate to show the differences in such a characteristic manner as was observed in the experiments with the other substances, especially the onset and the development of the effects are too slow to permit sharp comparisons. However, when the experiments are made simultaneously on a series of animals, the differences of the two groups are unmistakable and are perceived at a glance. The differences between the effects of intramuscular and subcutaneous injections of morphine presented themselves pronouncedly when both kinds of injections were practised on the same animals, of course after proper intervals. The following protocol will illustrate our statement:

Nov. 11.—Four rabbits received at about the same time 8 milligrams of morphine sulphate per kilo of animal. A and B received it intramuscularly; C and D subcutaneously. Within a few minutes after injection, the difference between the two groups was marked: A and B lying on their bellies, legs stretched out, heads resting on the table, and ears flat on back. C and D quiet, but head and ears erect and body not stretched out. Thirty or forty minutes later the difference in the behavior of the two groups still marked; the respirations, too, are much

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slower in the "intramuscular" group. An hour after the injections the difference between the two groups now less marked. Next day animals normal.

Nov. 15.—The same four animals again received 8 milligrams of morphine sulphate per kilo. But now the order was reversed: A and B were injected subcutaneously, C and D intramuscularly. Now C and D after eight minutes became droopy, lying on belly, etc., while A and B (now subcutaneous) show little or no effect. After twenty-four minutes all four animals were put on the floor: A and B hopped off to a corner, C and D remained on the spot where they had been put, in a droopy, somnolent condition. The difference in the behavior of the two groups was well marked, and the contrast in the behavior of each group in both experiments was indeed striking.

By the foregoing experiments we believe to have firmly established, at least for the substances we have experimented with, that absorption from intramuscular tissue is incomparably faster than from subcutaneous tissue. The effect of an intramuscular injection stands in value very near that of a direct injection into the circulation. That these injections into the muscles were not indeed intravenous injections, i. e., that, accidentally, each time the injection was made into a blood-vessel, needs hardly a refutation; the constancy of the results speaks sufficiently against their accidental nature. It could, however, be assumed that, the muscles being provided with many blood-vessels, an intramuscular injection is unavoidably an injection into the blood-vessels. Hence the rapid effect of an injection into the muscles was actually only the effect of an intravenous injection, and does not signify that a rapid absorption from the muscles into the blood-vessels takes place normally; in other words, our experiments do not prove that in the normal metabolic processes within the muscles the rate of absorption is different from that of the subcutaneous tissue. But the following facts speak against such an assumption. The rise of blood-pressure which was caused by an intramuscular injection of suprarenal extract was rarely as great as that which is caused

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by an intravenous injection of a similar dose. Furthermore, after repeated intramuscular injections a further injection by the same method would have no effect, while subsequent intravenous injections would still continue to cause a considerable rise of pressure. In the effect upon the pupil there is a distinct difference between the effects of intravenous and intramuscular injections. An intravenous injection of suprarenal extract would always cause a dilatation of the pupil, although of short duration, even when the superior cervical ganglion was intact, while we have not observed that an intramuscular injection ever caused any dilatation of the pupil on the unoperated side of the animal. Finally, the most striking differences between intramuscular and intravenous injections have been observed in our experiments with fluorescein. After intravenous injections, even with much smaller doses than those which we have employed in the present experiments, the effect is instantaneous: the conjunctivæ become orange-yellow, the pupils deep green, etc. within a few seconds after the injection. While with intramuscular injections the pupils did not become green until nearly half an hour after the injection, and the entire animal was by far not so deeply colored as after an intravenous injection.[8]

We therefore entertain the view that in intramuscular injections the fluid does not enter directly into the blood, but is at first deposited between the muscle fibres and is carried thence into the blood by some process of rapid absorption.

We have to add that, in our opinion, the difference between the absorption from intramuscular and subcutaneous tissues is only a matter of degree. We have seen in our previous experiments with fluorescein that one minute after a subcutaneous injection there was already fluorescein present in the serum of the blood. The difference consists only in the quantity which becomes absorbed in a unit of time, and the quantity absorbed is apparently so much larger from the intramuscular tissue than from the subcutis that it produces strikingly different results. Whether some substances are absorbed better from the intramuscular tissue than others, we cannot state for the present.

[8] S. J. Meltzer and John Auer, l. c.

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The fact that the difference between the effects of intramuscular and intravenous injection is much greater with fluorescein than with, for instance, curare, would speak for such a possibility. Future researches in this direction will throw light on this problem, as well as on many others which are bound to present themselves on the basis of our present investigation.

Why is the absorption from the muscles so much more favorable than from the subcutaneous tissue? Possibly because the unit of space in the muscles contains more blood and more blood-vessels, and is therefore capable of taking up more liquid injected into it in the same unit of time. Possibly the readiness of the blood-vessels of the muscles to respond with vaso-dilatation to local and reflex stimulation is also a factor in this process. It is possible also that the capillary walls of the muscular tissue are much more permeable than the capillary walls of the subcutaneous tissue. We shall, however, for the present not commit ourselves to any theory or enter even into a discussion of the merits of the factors concerned, as this would be prèmature. Not only has a new fact been observed, but a new field has been opened, which is bound to yield many more new facts capable of throwing more and better light upon the underlying causes. The application of the new point of view may be manifold. In physiology, for instance, we ought to learn in general, from the observations communicated in the preceding pages, that absorption from the tissues of the body is not a single, uniform process, but that the rate and the mode of absorption may vary with each tissue and organ. In particular we might study and come to understand the rapid interchange between the metabolic products of the muscles and the general circulation, the rapid effect which the catabolic products of the active muscles exert upon the mechanisms of circulation and respiration, and many other problems. In pathology we might study and come to understand whether and why infections and intoxications from the muscles occur less readily than from subcutaneous tissue, and why œdema of the muscles is a much less frequent phenomenon than œdema of the subcutaneous tissues. In the experimental studies of infection and immunity, the intramuscular incorporation of virus, toxin,

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and antitoxin should present a distinct method of study, and the results should be compared with those of other methods in vogue. In pharmacology the method of intramuscular injection should receive special attention. The action of pharmacological products which are effective in small doses should be tested by intramuscular injection with regard to the rapidity of action as well as the duration of the effect. The permissible dosage should be established. We have seen above that a dose of curare, for instance, which was absolutely harmless in subcutaneous application, was nearly fatal when given intramuscularly. In therapeutics, intramuscular injection might offer the advantages of an intravenous administration without its dangers. On the other hand, a combination of intramuscular and subcutaneous injections might ensure rapidity and intensity together with long duration of action.

EXPLANATION OF FIGURES.

The line on each figure represents variations in blood-pressure written by the mercury manometer. Base lines and time markings were omitted in printing the curves. The tracings were obtained from the carotid artery of rabbits after subcutaneous, intraperitoneal, and intramuscular injections of commercial adrenalin chloride (1:1000).

The first three figures were obtained in previous studies on the influence of adrenalin on the blood-pressure, administered by subcutaneous injection. (The time was marked in five seconds.)

PLATE I.

Fig. 1 represents a part of such a tracing; twenty-eight minutes passed before the elevation reached the original level. Only two curves with such a rise were obtained in an extended series of experiments.

Figs. 2 and 3 were obtained consecutively on the same animal. Fig. 3 was obtained by intravenous injection of 0.3 cc. Fig. 2 was obtained by an injection of 0.4 cc. in the region of the left pectoral muscle. As the autopsy revealed no injury to a blood-vessel, it was then taken for an exceptional effect of a "subcutaneous" injection. It was, however, apparently the effect of an intramuscular injection.

A cross on the curves marks the end of an injection.

Fig. 4.—1375 grams. 1 cc. subcutaneously, upper abdomen. *First* injection.

Fig. 5.—Same animal. *Second* injection; 0.8 cc. intramuscularly, left back, lumbar region.

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PLATE II.

Fig. 6.—2500 grams. *First* injection; 1 cc. subcutaneously, left upper abdomen.

Fig. 7.—Same animal. *Second* injection; 0.8 cc. intramuscularly, left back, lumbar region.

Fig. 8.—1500 grams. *First* injection; 0.6 cc. subcutaneously, abdomen.

Fig. 9.—Same animal. *Second* injection; 0.6 cc. intramuscularly, left back, lumbar muscles.

Fig. 10.—Same animal. *Third* injection; 0.6 cc. subcutaneously, right upper abdomen.

(Note that Figs. 8, 9, and 10 are consecutive.)

PLATE III.

Fig. 11.—1320 grams. Curare; artificial respiration; 0.8 cc. intraperitoneally through catheter in abdominal wall. *First* injection.

Fig. 12.—Same animal. *Second* injection; 0.8 cc. intramuscularly, left back, lumbar region.

Fig. 13.—2045 grams. *First* injection; 0.8 cc. intraperitoneally through cut in abdominal wall.

Fig. 14.—Same animal. *Second* injection; 0.8 cc. intramuscularly, left back, lumbar region.

Fig. 15.—1540 grams. Curare; artificial respiration; 1 cc. intramuscularly, left back, lumbar region. *First* injection.

Fig. 16.—Same animal. *Second* injection; 1 cc. intraperitoneally, through catheter.

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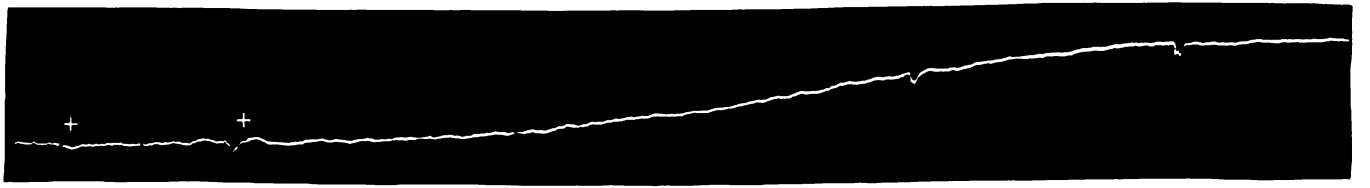
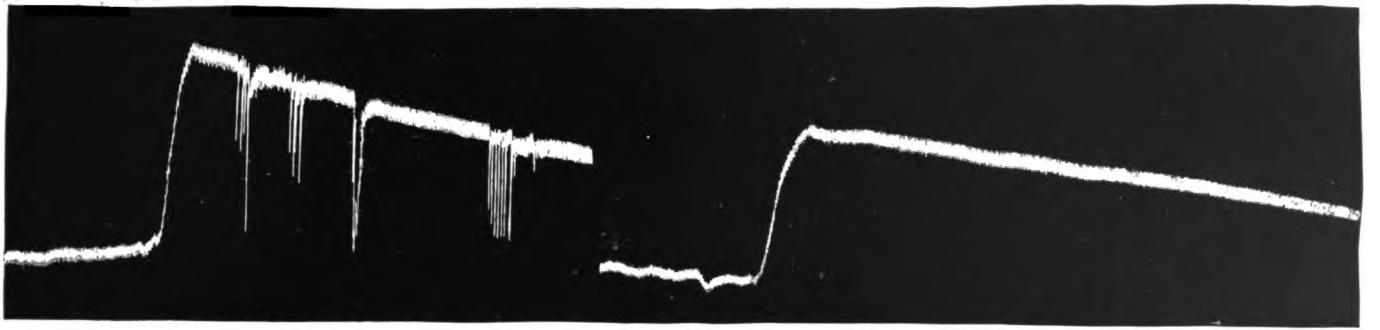


Subcutan., 0.8 cc. Massage.

FIG. 1 × ⅓.

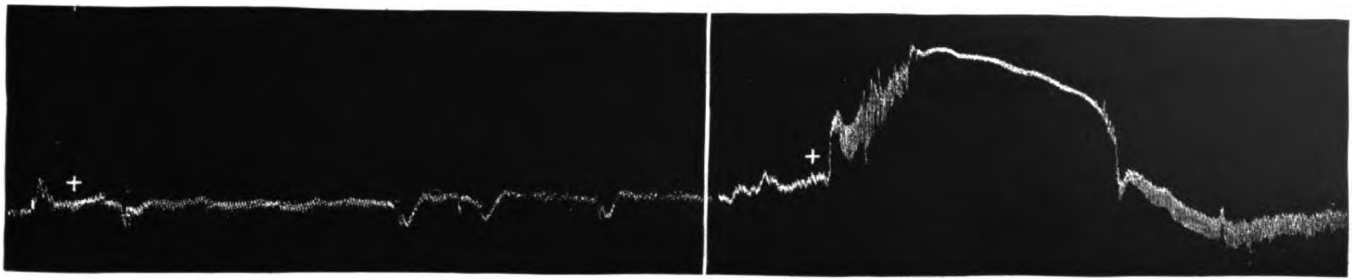


Subpectoral.
0.4 cc.

FIG. 2.

Intravenous.
0.3 cc.

FIG. 3.



Subcutan., 1 cc.
4.17.

FIG. 4 × ⅛.

Intramuscular, 0.8 cc.
4.26½.

FIG. 5 × ⅛.

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Subcutan., 0.6 cc. Struggle.
11.21.

FIG. 10.

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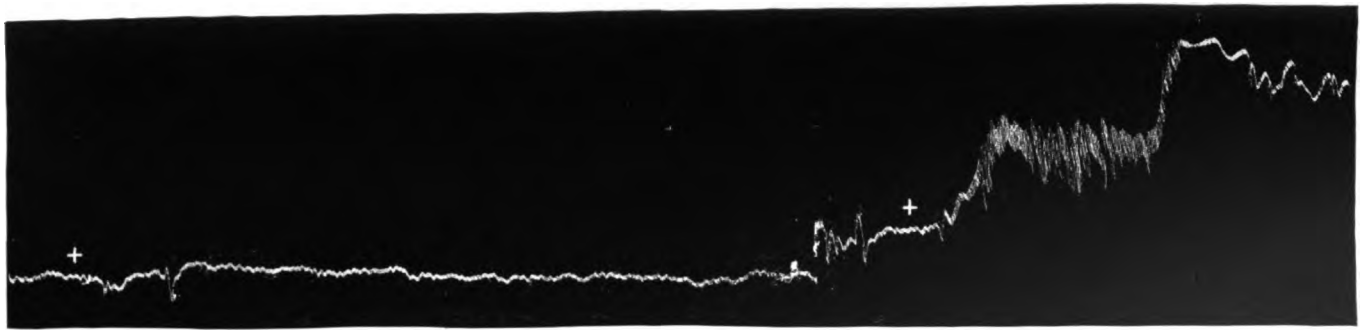
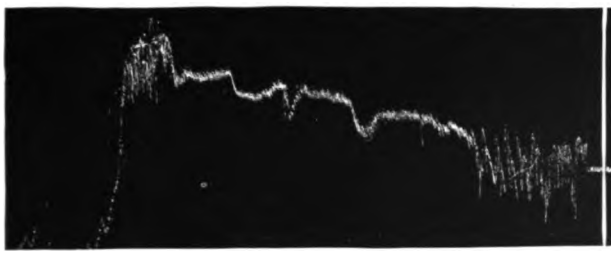


Intraperitoneal, 0.8 cc.
3.55. FIG. 13.

Intramuscular, 0.8 cc.
4.16. FIG. 14.



Intramuscular, 1 cc.
5.10. FIG. 15 × 1/6.

Intraperitoneal, 1 cc.
5.27. FIG. 16 × 1/6.

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VII. PLATE III.

Intraperitoneal, 0.8 cc.

Fig. 11

Intravascular, 0.8 cc.

Fig. 12.

NZYMES OF INFLAMMATORY UDATES.

IE L. OPIE, M.D.

te for Medical Research, New York.)

: in the fluids of the body when rgo solution through the agency of und or engulf them. Much study tors which influence phagocytosis tion has been given to the process

in pus was demonstrated by Eich- later confirmed by Maixner [2] and with a variety of diseases char- accumulation of pus, such as em- abscess, epidemic cerebro-spinal xner found pepton in the urine. the urine during the resolution of ng that the amount of pepton a relation to the number of cells s more abundant in the cellular g serum or in the filtrate from such led the conclusion that living pus mically or mechanically binding ymptom of the destruction of pus

ence of pepton in purulent sputum, ntly unpublished observations of at a glycerine extract of purulent

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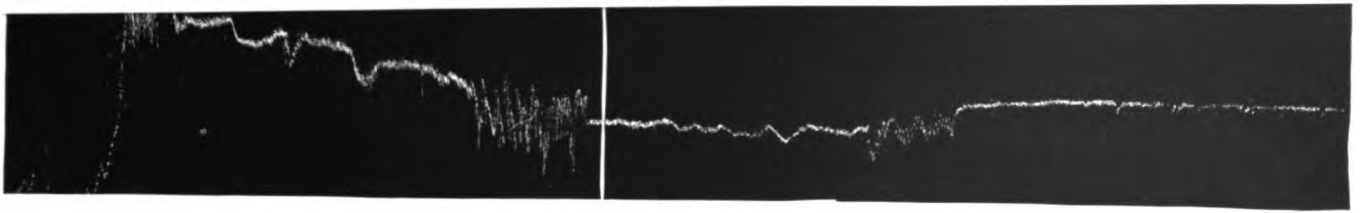

Intramuscular, 1 cc.
5.10. FIG. 15 × ⅙.

Intraperitoneal, 1 cc.
5.27. FIG. 16 × ⅙.

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ENZYMES AND ANTI-ENZYMES OF INFLAMMATORY EXUDATES.

By EUGENE L. OPIE, M.D.

(*From the Rockefeller Institute for Medical Research, New York.*)

Many substances insoluble in the fluids of the body when injected into the tissues undergo solution through the agency of phagocytic cells which surround or engulf them. Much study has been devoted to the factors which influence phagocytosis but comparatively little attention has been given to the process of intracellular digestion.

The occurrence of pepton in pus was demonstrated by Eichwald [1] as early as 1864, and later confirmed by Maixner [2] and Hofmeister.[3] In association with a variety of diseases characterized by formation and accumulation of pus, such as empyæma, purulent peritonitis, abscess, epidemic cerebro-spinal meningitis, and pyelitis, Maixner found pepton in the urine. Pepton was also observed in the urine during the resolution of croupous pneumonia. Finding that the amount of pepton present in purulent fluid bore a relation to the number of cells which were present, and was more abundant in the cellular sediment than in the overlying serum or in the filtrate from such an exudate, Hofmeister reached the conclusion that living pus cells have the power of chemically or mechanically binding pepton, and peptonuria is a symptom of the destruction of pus cells.

To explain the constant presence of pepton in purulent sputum, Kossel [4] cites hitherto apparently unpublished observations of Fr. Müller, who had found that a glycerine extract of purulent

[1] *Würzb. med. Zeit.*, 1864, 335. Cited by Hofmeister.
[2] *Prager Vierteljahrschrift*, cxliii, 78. Cited by Hofmeister.
[3] *Zeit. für phys. Chem.*, 1880, iv, 253, 268.
[4] *Zeit. für klin. Med.*, 1888, xiii, 149.

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phthisical sputum is capable of digesting in a weakly alkaline medium fibrin and coagulated albumin if decomposition is prevented. Other purulent sputa have the same power, but purely mucous sputum and the sputum of pneumonia before the crisis do not exhibit it. As soon as crisis occurs and the sputum has assumed a white pus-like appearance, the presence of a ferment is demonstrable. In the fresh pus obtained from an abscess, a similar ferment can be demonstrated, but is absent in the thin pus-like fluid of a cold abscess. Achalme [5] has studied the effect of pus upon gelatin, fibrin, coagulated egg-albumin, and casein, and has found that it is capable of liquefying or dissolving these substances. Similar observations have been made by Leber.[6]

Ascoli and Mareschi [7] subjected the cellular sediment of sterile exudates, obtained by injecting aleuronat into the peritoneal cavity, to autolysis under chloroform, and determined before and after digestion, by means of the Kjeldahl method, the amount of nitrogen not precipitable by tannin. A well marked digestion occurred but was absent when the cellular sediment had been previously heated to 60° C. for three hours.

Attempts to study the autolysis of fluids withdrawn from the serous cavities of individuals suffering with peritoneal or pleural effusions have given inconstant results. Estimating by means of the Kjeldahl method the amount of proteid converted by digestion into non-coagulable nitrogen-containing substances, Umber [8] showed that the peritoneal fluid from two patients suffering with abdominal tumors underwent autolysis. In ascitic fluid from five patients and in pleural exudates from two, Schütz [9] found by the same method no measurable destruction of proteids. The fluid in two instances contained a considerable number of leucocytes. The autolysis of exudates withdrawn from serous cavities of twelve patients suffering with hydro-

[5] *Compt. rend. de la Soc. de Biol.*, 1899, li, 568.

[6] *Ueber die Entstehung der Entzündung*, Leipzig, 1891.

[7] *Eleventh Italian Congress for Internal Medicine*, Pisa, October 27–31, 1901. Ref. Maly's *Jahresber.*, 1902, xxxii, 291.

[8] *Münchener med. Woch.*, 1902, xlix, 1169.

[9] *Cent. für inner. Med.*, 1902, xxiii, 1161.

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thorax, pleurisy, and ascites has been studied by Zak.[10] In one-half of the cases, including two cases of pleurisy, no autolysis was demonstrable, while in only four instances was there noteworthy digestion of coagulable albumin. Zak found no relation between the abundance of cells and the degree of autolysis.

Methods.—The present series of experiments was undertaken with the purpose of studying the capacity for proteolytic digestion possessed by inflammatory exudates at various stages. Exudates, obtained by injecting suspensions of plasmon or of aleuronat into the pleural cavities of dogs or rabbits, were, with aseptic precautions, removed from the animal killed by bleeding or were withdrawn from the etherized animal by aspiration. A suspension of plasmon in ten times its weight of 0.5 % potassium hydroxide was at first used, but aleuronat meal in from seven to ten times its weight of water, to which was added 3 % of starch, proved more useful. Of this suspension 10 cubic centimeters were injected into a pleural cavity. The exudate removed at the end of from one to three days was introduced in carefully measured quantities into small flasks and diluted with four times its volume of 0.85 % salt solution. In most instances 1 cubic centimeter of toluol was added to each flask, which was then placed in the thermostat at 37° C. At the end of two or more days the flasks were removed and their contents were heated to 100° C., after acidification with acetic acid.

Complete precipitation of the coagulable proteids was at first effected with some difficulty, but was later much facilitated by the addition of an equal volume of 16 % solution of magnesium sulphate. Should precipitation be incomplete, filtration is difficult and the filtrate is turbid. By adjusting the reaction to slight acidity with acetic acid (1 %), or with sodium hydroxide ($\frac{1}{10}$ N.) if acid has been added in excess, albumin still in solution may be coagulated by heat and again removed by filtration. The filtrate from the coagulum repeatedly washed was received directly into a Kjeldahl flask and the amount of nitrogen present was estimated by the Kjeldahl method.

[10] *Wiener klin. Woch.*, 1905, xviii, 376.

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The filtrate from an equal amount of exudate diluted in the same way and immediately coagulated by heat served as a control. The difference between the autolyzed exudate and its control represents in terms of $\frac{1}{10}$ N. sulphuric acid the nitrogen of the coagulable proteid converted by digestion into soluble form. I am indebted to Dr. Flexner and to Dr. P. A Levene for numerous suggestions which have aided me in the application of the chemical methods employed.

Autolysis of Sterile Inflammatory Exudates.—In the following experiments, sterile exudates, obtained by injecting a suspension of aleuronat into the right pleural cavity of dogs, were removed aseptically and kept in sterile flasks without addition of toluol or other antiseptic for forty-eight hours at body temperature. That no autolysis had occurred during this time is shown by the following figures which represent in terms of $\frac{1}{10}$ N. acid the nitrogen of substances in solution after coagulation by heat.

EXPERIMENT 1.—Reddish turbid fluid containing many leucocytes and a considerable number of red blood corpuscles was removed from the right pleural cavity of a dog twenty-four hours after the injection of aleuronat. Nitrogen present in the filtrates from the coagulated fluid is represented by c. c. of $\frac{1}{10}$ N. acid.

5 c. c. exudate at 37° C. 2 days	3.1 c. c.
Control	3.7 "

EXPERIMENT 2.—Exudate was removed forty-eight hours after the injection of aleuronat into the right pleural cavity of a dog. The exudate contained 6.5 % of cells.

5 c. c. exudate at 37° C. 2 days	3.05 c. c.
Control	3.75 "

In both experiments less nitrogen was found in the control subjected to immediate coagulation than in the flask kept forty-eight hours at body temperature. The significance of this fact, confirmed by subsequent experiments, will be discussed later.

In similar experiments the exudates obtained from rabbits by injecting aleuronat into the pleural cavity were used. The diluted exudate was kept at body temperature four days.

EXPERIMENT 3.—Exudate was removed twenty-four hours after the injection of 10 c. c. of aleuronat suspension into the right pleural cavity of a rabbit. The cells present formed 6 % of its volume.

3 c. c. exudate at 37° C. 4 days (aseptic)	0.7 c. c.
Control	0.9 "

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EXPERIMENT 4.—Exudate was removed two days after the injection of 5 c. c. of aleuronat suspension. Cells form 5.5 % of the volume.

3 c. c. exudate at 37° C. 4 days (with toluol)	1.65 c. c.
Control	1.7 "

EXPERIMENT 5.—Exudate was removed three days after the injection of 10 c. c. of aleuronat. Cells form 2.5 % of the volume.

1 c. c. exudate at 37° C. 4 days (aseptic)	2.10 c. c.
Control	1.55 "

No autolysis was obtained except with the exudate removed three days after the injection of the inflammatory irritant. Since it was suspected that autolysis occurred only after the death of cells contained in the exudate, longer periods of digestion were considered necessary, and at the same time the effect of toluol, which presumably destroys the cells, was studied. A considerable number of flasks containing equal quantities of exudate, some aseptically prepared, others prepared with toluol, were kept at body temperature for varying periods as indicated below.

EXPERIMENT 6.—An exudate containing 4 % cells was obtained twenty-four hours after injecting 10 c. c. aleuronat suspension into the right pleural cavity of a dog. Each flask contained 3 c. c. exudate diluted as in previous experiments.

	Aseptic.	With toluol.
Control	2.0 c. c.	
At 37° C. 2 days	2.0 "	1.3 c. c.
" " " 4 "	2.1 "	2.15 "
" " " 6 "	1.7 "	2.5 "
" " " 8 "	1.7 "	2.55 "

EXPERIMENT 7.—An exudate containing 2.5 % cells was removed from the right pleural cavity of a dog three days after the injection of 10 c. c. aleuronat. Each flask contained 3 c. c. exudate.

	Aseptic.	With toluol.
Control	7.9 c. c.	7.7 c. c.
At 37° C. 2 days	7.8 "	7.7 "
" " " 4 "	9.9 "	10.25 "
" " " 6 "	12.9 "	10.9 "

Exudates removed both from the dog and from the rabbit one or two days after the injection of aleuronat underwent little if any digestion, while that removed three days after injection autolyzed to an appreciable though slight extent. The presence of toluol did not very materially affect the result in either case, and greatly facilitates the performance of the experiment, since

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it obviates the necessity of preserving the strictest asepsis when, as in subsequent experiments, centrifugalization of the exudate is necessary. In the preceding experiments, cultures made from each flask at the end of digestion remained sterile.

It is noteworthy that Schütz, attempting to autolyze exudates obtained by tapping the pleural or peritoneal cavities, obtained negative results, while those of Zak were inconstant. Zak found no relation between autolysis and the cellular contents of the exudate.

Autolysis of Leucocytes.—In several instances the quantity of cells in the exudates employed in the preceding experiments was estimated by means of the hæmatocrit and varied by volume from 2.5 % to 6.5 %. There was no relation between the proportion of cells and the occurrence of autolysis. In the exudates obtained from rabbits, those removed one and two days after injection contained respectively 6 % and 5.5 % of cells, while the exudate which was removed on the third day and which underwent slight digestion contained 2.5 %. In order to determine if autolysis affected only the cells, in the following experiments cells of an exudate twenty-four hours after injection of aleuronat were separated from the serum by centrifugalization and suspended in nineteen times their volume of .85 % solution of sodium chloride (5 % suspension). The suspension was diluted with four times its volume of salt solution and subjected to autolysis during five days. An equal amount of serum was kept during the same time at 37° C. and compared with a control flask coagulated immediately.

EXPERIMENT 8.—In 5 c. c. of a 5 % suspension of cells and in 5 c. c. of serum, obtained from an exudate removed twenty-four hours after the injection of aleuronat into the pleural cavities of a dog, the amount of nitrogen contained in substances not coagulable by heat before and after digestion is represented in terms of $\frac{1}{10}$ N. acid by the following figures:

5 c. c. cells at 37° C. 5 days	2.35 c. c.
Control	.75 "
5 c. c. serum at 37° C. 5 days	3.7 "
Control	4.15 "

With cells alone well marked autolysis occurred, although with the entire exudate removed at the same stage of inflam-

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mation and containing approximately the same number of cells, none was demonstrable (e.g., Experiment 2, in which the percentage of cells in the exudate used was 6 %). It is noteworthy that the serum kept at 37° C. for five days, like the whole exudate in previous experiments, contained less nitrogen in soluble form than the control coagulated immediately.

Anti-enzymotic Action of the Serum of Sterile Inflammatory Exudates.—The experiment just described has suggested that the serum of the inflammatory exudate inhibits the autolysis of the leucocytes which it contains. Obviously this possibility may be tested by separating, by centrifugalization, cells and serum and digesting separately and after recombining.

In the following experiment, cells from an exudate removed from the pleural cavity twenty-four hours after the injection of aleuronat were suspended in nine times their volume of salt solution. In order to test the autolysis of these cells, 5 cubic centimeters of the suspension were diluted with 20 cubic centimeters of salt solution. To the same quantity of cells was added 1 cubic centimeter, and in another flask 5 cubic centimeters, of the serum of the exudate, and in every instance the total volume of fluid was brought to 25 cubic centimeters by the addition of salt solution. In another flask 5 cubic centimeters of serum, diluted with 5 cubic centimeters of salt solution, were heated for twenty minutes in boiling water; to this coagulated serum were added 5 cubic centimeters of the suspension of cells. After addition of toluol the various mixtures were kept at 37° C. for five days. The nitrogen in uncoagulable compounds, contained in 5 cubic centimeters of serum from the exudate diluted to 25 cubic centimeters and allowed to remain in the thermostat for the same time, was, for the sake of comparison, estimated by the usual method.

EXPERIMENT 9.—Cells and serum, obtained from an exudate removed from the pleural cavities of a dog twenty-four hours after the injection of aleuronat, were tested as follows:

5 c. c. cells at 37° C. 5 days	8.2	c. c.
Control	1.3	"
Digestion	6.9	"

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5 c. c. serum at 37° C. 5 days	4.35	c. c.
5 c. c. cells + 1 c. c. serum at 37° C. 5 days	3.45	"
Uncoagulable nitrogen in 5 c. c. cells (1.3 c. c.) and 1 c. c. serum (approximately 0.87 c. c.) represented by	2.17	"
Digestion	1.28	"
5 c. c. cells + 5 c. c. serum at 37° C. 5 days	7.75	" *
Uncoagulable nitrogen in 5 c. c. cells (1.3 c. c.) and 5 c. c. serum (approximately 4.35 c. c.) represented by	5.65	"
Digestion	2.1	"
5 c. c. cells + 5 c. c. coagulated serum at 37° C. 5 days	30.65	"
Uncoagulable nitrogen in 5. c. c cells and 5 c. c. serum represented by	5.65	"
Digestion	25.00	"

* Culture from this flask showed bacterial contamination; the flask had been insecurely closed and the toluol used had escaped.

The autolysis of the leucocytes contained in the suspension is inhibited by the addition of serum, so that the nitrogen derived by digestion from coagulable proteids is diminished to less than one-third. When, however, the serum is heated to approximately 100° C. no inhibition occurs and digestion proceeds far beyond that referable to autolysis of cells. The intracellular ferments, the action of which is now unrestrained, attacks the proteids furnished by the heated serum.

The following experiments confirm the results just described:

EXPERIMENT 10.—Cells obtained twenty-four hours after the injection of aleuronat into the pleural cavities of a dog were suspended in nineteen times their volume of salt solution.

5 c. c. cells at 37° C. 5 days	4.55	c. c.
Control	2.05	"
Digestion	2.5	"
5 c. c. serum at 37° C. 5 days	3.85	"
Control (coagulated immediately)	4.5	"
Digestion	——	
5 c. c. cells + 5 c. c. serum at 37° C. 5 days	5.4	"
Uncoagulable nitrogen in 5 c. c. cells (2.05 c. c.) and 5 c. c. serum (4.5 c. c.) represented by	6.55	"
Digestion	——	

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5 c. c. cells + 5 c. c. coagulated serum at 37° C. 5 days....	14.2 c. c.
Uncoagulable nitrogen in 5 c. c. cells and 5 c. c. serum represented by.....	6.55 "
Digestion.....	7.65 "

EXPERIMENT 11.—Cells from an exudate removed from the pleural cavities of a dog three days after the injection of aleuronat were suspended in nine times their volume of salt solution.

5 c. c. cells at 37° C. 5 days.....	9.3 c. c.
Control.....	3.6 "
Digestion.....	5.7 "
5 c. c. serum at 37° C. 5 days.....	6.5
Control (coagulated immediately).....	7.25 "
Digestion.....	——
5 c. c. cells + 1 c. c. serum at 37° C. 5 days.....	10.2 "
Uncoagulable nitrogen in 5 c. c. cells (3.6 c. c.) and 1 c. c. serum (1.45 c. c.) represented by.....	5.05 "
Digestion.....	5.15 "
5 c. c. cells + 1 c. c. coagulated serum at 37° C. 5 days.....	11.05 "
Uncoagulable nitrogen in 5 c. c. cells and 1 c. c. serum represented by.....	5.05 "
Digestion.....	6.00 "
5 c. c cells + 5 c. c. serum at 37° C. 5 days.....	10.95 "
Uncoagulable nitrogen in 5 c. c. cells (3.6 c. c.) and 5 c. c. serum (7.25 c. c.) represented by	10.85 "
Digestion.	.1 "
5 c. c. cells + 5 c. c. coagulated serum at 37° C. 5 days	23.1 "
Uncoagulable nitrogen in 5 c. c. cells and 5 c. c. serum represented by.....	10.85 "
Digestion.....	12.25 "

It is noteworthy that the inhibitory action of the serum of an exudate used in the last experiment and removed three days after the injection of the imflammatory irritant is less than that of the serum from the exudates employed in the two preceding experiments and withdrawn only twenty-four hours after injection. One cubic centimeter of the serum in this experiment had little effect upon the digestion of the cells mixed with it (digestion of cells alone, 5.7 cubic centimeters; of cells with 1

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cubic centimeter serum, 5.15 cubic centimeters), while in the preceding experiments the same amount of serum completely prevented or materially retarded digestion. This observation is in agreement with the fact already noted, that the whole exudate removed one or two days after the onset of inflammation undergoes little if any autolysis, while that of exudates three days old is considerable.

That the digestion of other proteids by enzymes contained in the leucocytes is inhibited by the serum of the exudate has been shown by several readily performed experiments in which gelatin and fibrin have been subjected to the action of cells and to cells with serum.

EXPERIMENT 12.—To test-tubes containing 6 c. c. 6 % gelatin was added 1 c. c. 20 % suspension of cells from an exudate obtained twenty-four hours after the injection of aleuronat. To a second gelatin tube were added 1 c. c. suspension of cells and 1 c. c. serum from the same exudate. After remaining twenty hours at 37° C., the gelatin containing cells alone remained liquid after cooling while that with cells and serum solidified.

EXPERIMENT 13.—To one of two tubes containing balanced quantities of fibrin obtained from dog's blood and cut into small pieces, were added 2 c. c. 20 % suspension of cells from an exudate removed two days after injection of aleuronat; to the second, 2 c. c. suspension and 2 c. c. serum from the exudate, the volume being made in both instances 8 c. c. with salt solution. At the end of forty-eight hours under toluol at 37° C. fibrin in the tube containing cells had almost completely disappeared, while that mixed with cells and serum was apparently unchanged.

Effect of Temperature upon the Anti-enzymotic Action of the Exuded Serum.—It has already been shown that the anti-enzymotic action exerted by the serum of a sterile exudate upon the proteolytic ferment of the leucocytes is destroyed by a temperature of 100° C. In order to determine at what point of an ascending scale the inhibitory power is destroyed, equal quantities of serum, diluted with an equal volume of salt solution, were heated to 45°, 55°, 65°, 75°, and 100° C. for one-half hour and then mixed with a suspension of cells, the entire mixture being brought to a volume of 25 cubic centimeters by addition of salt solution.

EXPERIMENT 14.—Cells from an exudate obtained three days after the injection of aleuronat into the pleural cavity of a dog were, after repeated washing

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suspended in nine times their volume of salt solution. Mixtures prepared as follows were kept in the thermostat at 37° C. for five days:

(5 c. c. cells	+	5 c. c. serum)	coagulated as control					8.45	c. c.
" "	+	"						9.9	"
" "	+	"	"	heated to	45°	C.		8.5	"
" "	+	"	"	" "	55°	C.		8.8	"
" "	+	"	"	" "	65°	C.		7.8	"
" "	+	"	"	" "	75°	C.		25.05	"
" "	+	"	"	" "	100°	C.		27.0	"

A temperature of 75° C. evidently suffices to destroy the anti-enzymotic action of the serum. Lower temperatures apparently cause a slight increase of the inhibition exerted by the serum, but the explanation of the slight change which occurs is not clear. It is not improbable that a temperature lower than 75° C. produces changes in the serum which exert an unfavorable influence upon the ferment or ferments contained in the leucocytes, or, as will subsequently appear, upon a proleolytic enzyme contained in the serum and destroyed perhaps by 65° C.

Effect of Reaction upon the Enzymes and Anti-enzymes of Sterile Inflammatory Exudates.—It was next considered desirable to study the influence of reaction upon this anti-enzymotic action of the serum of sterile exudates. This exuded serum is strongly alkaline to litmus. To mixtures of cells and serum, prepared as in previous experiments, was added sodium bicarbonate or acetic acid to the strength of 0.2%. For comparison the same amount of alkali or acid was added to similar mixtures of cells and coagulated serum.

EXPERIMENT 15.—Cells from an exudate removed twenty-four hours after the injection of aleuronat were suspended in nine times their volume of salt solution. Mixtures containing 5 c. c. of this suspension and 5 c. c. of serum were kept in the thermostat at 37° C. for five days.

	Cells + serum.	Cells + coagulated serum.
With 0.2 % sodium bicarbonate..	10.1 c. c.	35.05 c. c.
Reaction unchanged.....	12.9 "	24.0 "
With 0.2 % acetic acid.....	27.05 "	19.55 "

The leucocytes of the exudate are capable of digesting proteids in both an alkaline and in an acid medium, but are more efficient in the former. The inhibition exerted by the serum

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is favored by an increase of the normal alkalinity of this fluid but is completely lost with the acidity produced by 0.2 % acetic acid. In this acid medium there is, indeed, more digestion of proteid with unheated than with heated serum. The possibility that the serum of the exudate contains an enzyme active in an acid medium is suggested. The preceding experiment was repeated and at the same time to serum alone was added alkali or acid in the quantity previously used.

EXPERIMENT 16.—Cells obtained from an exudate three days after the injection of aleuronat were suspended in nine times their volume of salt solution. Mixtures containing 5 c. c. suspension of cells and 5 c. c. serum, 5 c. c. suspension and 5 c. c. coagulated serum, and 5 c. c. serum alone were prepared as follows and kept in the thermostat at 37° C. for five days:

	Cells + serum.	Cells + coagulated serum.	Serum.
With 0.2 % sodium bicarbonate..	8.0 c. c.	35.15 c. c.	6.25 c. c.
Reaction unchanged.....	9.9 "	27.0 "	4.6 "
With 0.2 % acetic acid.....	33.8 "	26.3 "	13.75 "

The nitrogen of uncoagulable substances contained in 5 c. c. of the serum before digestion is represented by 6.3 c. c. $\frac{1}{10}$ N. acid.

The experiment confirms the results previously obtained. An increased alkalinity favors the inhibition of the intracellular ferments, which are, when this inhibition is destroyed by heat, themselves most active in an alkaline medium. The addition of acid to a mixture of unheated serum and cells destroys the anti-enzymotic action of the serum, but in this acid medium digestion is more active than in a mixture of cells and serum of which the anti-enzymotic action has been destroyed by heat. This increased digestion is doubtless due to the action of a ferment contained in the serum and made active by the addition of acetic acid. For no proteolysis occurs when alkali is added to serum alone, and, when the reaction is unchanged, the same diminution of nitrogen in uncoagulable form, noted in preceding experiments, is found, but in an acid medium there is well marked digestion of proteid (represented by 13.75 minus 6.3 equals 7.45 cubic centimeters). The serum contains a ferment which, being destroyed by heat, explains the greater proteolysis obtained with cells and unheated serum than with

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cells and heated serum (represented by 33.8 minus 26.3 equals 7.5 cubic centimeters).

In the serum of the blood Delezenne and Pozerski [11] have demonstrated the existence of a proteolytic enzyme capable of exerting its action on gelatin and casein when the serum is treated with chloroform. Under ordinary conditions, they believe, anti-bodies which may be destroyed by chloroform inhibit the action of the ferment. Hedin [12] has more recently found in the blood serum of the ox a weak proteolytic enzyme which acts in an alkaline medium. The enzyme is precipitated with the globulin of the serum; anti-bodies which inhibit its action are, he finds, contained mainly in the albumin fraction of the serum.

Destruction of the anti-enzymotic action of the blood serum by acetic acid is in accord with observations of Levene,[13] who has shown that the resistance which uncoagulated egg-albumin and normal blood serum offer to digestion by an extract of fresh pancreatic tissue is overcome by exposing these substances to the action of acetic acid before addition of the extract.

Anti-enzymotic Action of the Serum of the Blood.—The well-known fact that certain anti-ferments are present in the normal blood serum has suggested the probability that the anti-body which in the serum of a sterile inflammatory exudate inhibits the activity of ferment in the leucocytes is derived from the serum of the blood. The action of blood serum of the dog was therefore compared with the serum of an exudate obtained by injecting aleuronat into the pleural cavity.

EXPERIMENT 17.—An exudate was obtained by injecting aleuronat into the pleural cavity of a dog, and the cells removed by centrifugalization were suspended in nineteen times their volume of salt solution. In order to increase the amount of digestible proteid, into each flask were put 5 c. c. of diluted blood serum coagulated by heating to 100° C. The carefully sealed flasks containing toluol were kept at 37° C. for five days. Blood serum was obtained by centrifugalization of the defibrinated blood.

(Cells + blood serum) coagulated as control.....	3.45 c. c.
Cells + coagulated blood serum at 37° C. 5 days.....	10.95 "

[11] *Compt. rend. de la Soc. de Biol.*, 1903, lv, 327, 690, 693.

[12] *Jour. of Phys.*, 1904, xxx, 195.

[13] *Jour. of Med. Research*, 1903, x, 217.

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Cells + coagulated blood serum + 5 c. c. exuded serum at 37° C. 5 days	8.5 c. c.
Cells + coagulated blood serum + 5 c. c. blood serum at 37° C. 5 days	8.25 "

When it is recalled that the serum of the exudate contains a considerable amount of nitrogen in a form not coagulable by heat, it is evident that inhibition of digestion is more marked than that indicated by these figures. The inhibitory action of blood serum and of serum from the inflammatory exudate is, it appears, approximately equal. In order to compare more accurately the effect of serum from the blood and from the exudate, another series of mixtures was prepared from the blood serum and exuded serum of an animal which had received an intrapleural injection of aleuronat.

EXPERIMENT 18.—Washed cells from an exudate obtained two days after the injection of aleuronat were suspended in nine times their volume of salt solution. Each flask contained 5 c. c. suspension of cells and 5 c. c. exuded serum or 5 c. c. blood serum, and was kept at 37° C. for five days.

(Cells + exuded serum) coagulated as control	6.4 c. c.
Cells + exuded serum at 37° C. 5 days	7.9 "
Cells + coagulated exuded serum at 37° C. 5 days	21.55 "
(Cells + blood serum) coagulated as control	6.25 c. c.
Cells + blood serum at 37° C. 5 days	8.35 "
Cells + coagulated blood serum at 37° C. 5 days	31.6 "

The experiment demonstrates that the anti-enzymotic action exhibited by the serum of the exudate is exerted by the serum of the blood as well, and in the case of a sterile exudate two days after the onset of inflammation in approximately the same degree.

Anti-enzymotic Action of the Serum of Bacterial Exudates.—An accidental infection of the pleural cavity of the dog afforded an opportunity for the study of an exudate containing bacteria. Following aspiration of the right pleural cavity twenty-four hours after the injection of aleuronat, breathing became laborious and the animal appeared sick. By aspiration three days later, about 150 cubic centimeters of opaque grayish-white fluid were withdrawn from the right pleural cavity. Cultures from the

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exudate showed the presence of streptococcus pyogenes in immense numbers, accompanied by a few colonies of a coccus which proved to have the cultural characters of staphylococcus pyogenes albus. Cover-slip preparations from the exudate contained cocci in great numbers, situated largely outside of the leucocytes. The animal died shortly after the removal, by aspiration, of 150 cubic centimeters of exudate.

EXPERIMENT 19.—Cells from the exudate containing streptococcus pyogenes and staphylococcus pyogenes albus were separated from the serum by centrifugalization and suspended in approximately nine times their volume of salt solution. Mixtures of cells and serum were made, and to each were added 5 c. c. toluol. Cultures made from each flask at the end of incubation remained sterile.

5 c. c. cells at 37° C. 5 days	6.05 c. c.
Control	2.85 "
Digestion	3.20 "
5 c. c. serum at 37° C. 5 days	8.25 "
Control (coagulated immediately)	7.7 "
5 c. c. cells + 1 c. c. serum at 37° C. 5 days	7.25 "
Uncoagulable nitrogen in 5 c. c. cells (2.85 c. c.) and 1 c. c. serum (1.54 c. c.) represented by	4.39 "
Digestion	2.86 "
5 c. c. cells + 1 c. c. coagulated serum at 37° C. 5 days	8.65 "
Uncoagulable nitrogen in 5 c. c. cells and 1 c. c. serum represented by	4.39 "
Digestion	4.26 "
5 c. c. cells + 5 c. c. serum at 37° C. 5 days	12.15 "
Uncoagulable nitrogen in 5 c. c. cells (2.85 c. c.) and 5 c. c. serum (7.7 c. c.) represented by	10.55 "
Digestion	1.60 "
5 c. c. cells + 5 c. c. coagulated serum at 37° C. 5 days	21.85 "
Uncoagulable nitrogen in 5 c. c. cells and 5 c. c. serum represented by	10.55 "
Digestion	11.30 "

The anti-enzymotic action exhibited by the serum of a sterile inflammatory exudate has been demonstrable in an exudate containing bacteria in considerable number. Inhibition of en-

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zymotic action is, however, less complete than in many of the experiments previously recorded. This difference may be partially referable to the fact that the leucocytes in this experiment were less thoroughly washed with salt solution than were those used in other experiments.

In the following experiment an exudate obtained by injecting bacillus coli into the pleural cavity of a dog was used. The serum of the exudate exerted an inhibitory effect upon the cellular ferments, but in the amount previously used the serum failed to prevent completely digestion.

EXPERIMENT 20.—Two entire cultures of bacillus coli grown forty-eight hours on agar-agar surfaces 7 × 14 cm. were injected into the right pleural cavity of a dog. Twenty-two hours later 30 c. c. of turbid reddish fluid were withdrawn by aspiration. Carefully washed cells in nine times their volume of salt solution were subjected to autolysis under toluol in the following mixtures:

5 c. c. cells at 37° C. 5 days	7.1	c. c.
Control	2.25	"
Digestion	4.85	
5 c. c. cells + 5 c. c. serum at 37° C. 5 days	7.35	"
(5 c. c. cells + 5 c. c. serum) coagulated immediately as control	4.55	"
Digestion	2.80	"
5 c. c. cells + 5 c. c. coagulated serum at 37° C. 5 days	18.7	"
(5 c. c. cells + 5 c. c. serum) coagulated immediately as control	4.55	"
Digestion	14.15	"

Cultures made from each mixture at the end of incubation remained sterile. although the exudate on removal from the pleural cavity contained bacillus coli, recovered by culture.

Changes Produced by Incubation in the Serum of Inflammatory Exudates.—A fact repeatedly observed during the course of these experiments requires some notice. When exudates removed one or two days after the onset of inflammation are subjected to autolysis, less nitrogen is demonstrable in the uncoagulable filtrate after digestion for from two to five days than in the control prepared by coagulating the fresh exudate. This fact is illustrated by Experiments 1, 2, 3, 4, 6, 7, in which this difference is at times considerable, representing in Experiments 1, 2, 3, and

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6 (aseptic digestion) from one-fifth to one-eighth of the nitrogen present in the filtrate from the fresh exudate. Subsequent experiments (Nos. 8, 10, 11, and 14) show that this difference is referable to the serum alone.

	Uncoagulable nitrogen in fresh serum of exudate represented by	Uncoagulable nitrogen in serum of exudate after incubation at 37° C. represented by
Experiment 8....	4.15 c. c.	3.7 c. c.
" 10....	4.5 "	3.85 "
" 11....	7.25 "	6.5 "
" 14....	6.3 "	4.6 "

In Experiment 10, a mixture of cells and serum confirmed the result obtained with serum alone. An exception to the facts just described is afforded by Experiment 14, in which the exudate was removed from the pleural cavity three days after the onset of inflammation, and by Experiment 19, in which the serum of the exudate contained streptococci in great numbers. The explanation of this disappearance of uncoagulable nitrogen is not evident. There is little probability that nitrogen has escaped as ammonia formed by digestion. It is not inconceivable that proteids have been less completely coagulated in the control than in the specimen incubated at 37° C., but there is no direct evidence of such a difference. Transformation of soluble nitrogen-containing substances into a form precipitable by heat is obviously not demonstrable by the methods employed.

Anti-enzymes.—Anti-bodies to several enzymes have been found in the normal blood serum. Hammarsten and Röden [14] demonstrated that the blood serum of the horse inhibits the action of lab-ferment and thus prevents coagulation of milk. The phenomenon has been studied by Briot,[15] Morgenroth,[16] and Fuld and Spiro.[17] The anti-tryptic action of the blood serum was first noted by M. Hahn [18] and later studied by a considerable

[14] *Upsala Läkareforenings forhandlingar*, xxii, 546; Ref. Maly's *Jahresb.*, 1887, xvii, 160.

[15] *Compt. rend. de l'Acad. des Sciences*, 1899, cxxviii, 1359.

[16] *Cent. für Bakt.*, 1899, xxvi, Abt. i, 349; 1900, xxvii, Abt. i, 721.

[17] *Zeit. für phys. Chem.*, 1900, xxxi, 132.

[18] *Berliner klin. Woch.*, 1897, xxxiv, 499.

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number of observers, Landsteiner,[19] Glaessner,[20] and Cathart [21] having sought to determine to what fraction of the serum the anti-body is attached. Ascoli and Bezzola [22] have described an increase of the anti-tryptic action of the blood serum during the early stage of pneumonia, followed by a gradual return to normal after the crisis. Delezenne [23] maintains that the anti-tryptic action of the blood serum is referable to an anti-enterokynase preventing the amboceptor-like action of enterokynase. Bayliss and Starling,[24] believing with Pawlow that enterokynase is a ferment converting trypsinogen into trypsin, oppose the view of Delezenne and furnish evidence to prove the existence of a true anti-trypsin. In a limited number of individuals they find that the serum of the rabbit contains anti-enterokynase.

Hedin [25] has demonstrated the presence in the spleen of two ferments, one acting in an acid the other in an alkaline medium; in the serum of the ox he has found an anti-body inhibiting the action of the latter and attached mainly to the albumin fraction of the serum.

The foregoing experiments show that the ferments contained in the leucocytes are incapable of acting in the serum of the exudates employed. The activity of these ferments is apparently limited to the cells which produce them. Solid particles ingested by the cells and thus separated from the serum are acted upon by these ferments. By phagocytosis substances insoluble in the fluids of the body are brought into contact with powerful ferments capable if unchecked of destroying tissues. Destruction of tissue occurs within an abscess when with the formation of pus, as the result perhaps of loss of anti-enzymotic action, fibrin and necrotic tissue undergo solution. In the later stages of sterile inflammation and with bacterial infection of a serous cavity some loss of anti-enzymotic power has been demonstrable.

[19] *Cent. für Bakt.*, 1900, xxvii, Abt. i, 357.
[20] Hofmeister's *Beiträge zür chem. Phys.*, 1903, iv, 79.
[21] *Jour. of Phys.*, 1904, xxxi, 497.
[22] *Berliner klin. Woch.*, 1903, xl, 391.
[23] *Compt. rend. de la Soc. de Biol.*, 1903, lv, 132.
[24] *Jour. of Phys.*, 1905, xxxii, 129.
[25] *Ibid.*, 1904, xxx, 155.

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It is not improbable that the number of anti-enzymes is greater than that now recognized. Facts already known suggest that such bodies accompany many ferments, holding them in check and limiting their action to those situations in which their proper function is accomplished.

CONCLUSIONS.

The serum of an inflammatory exudate has the power of inhibiting the action of proteolytic ferments contained in the leucocytes. This anti-enzymotic power is possessed by the blood serum from which it doubtless passes into the exudate.

In the later stages of inflammation there is some diminution of this anti-enzymotic action.

The anti-body contained in the serum is destroyed by a temperature of 75° C.

The proteolytic ferments of the leucocytes act both in an acid and in an alkaline medium but are most efficient in the latter. The anti-enzymotic action of the serum is favored by an alkaline reaction, but is completely lost in an acid medium.

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Reprinted from The Journal of Experimental Medicine, 1905.

THE ARTIFICIAL ANASTOMOSIS BETWEEN THE PORTAL VEIN AND THE VENA CAVA INFERIOR—ECK'S FISTULA.[1]

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Plates VI-VIII.

It is the purpose of the following paper to discuss briefly the methods which have been used in the operation for directing the blood of the portal vein into the vena cava, and to describe a new instrument for cutting the opening between the veins. The operation is named after N. V. Eck (1), who conceived the idea of establishing an artificial anastomosis between the portal vein and the vena cava, in order to relieve the congestion of the portal system in cirrhosis of the liver. Never attempted upon the human being, the operation performed on dogs has yielded a great part of the experimental facts concerning the normal physiology of the liver, and still offers an invaluable method for approaching many problems of metabolism. I shall leave aside for the present the question of the changes in the animal economy which follow the diversion of the portal blood current, simply appending a list of the literature bearing upon the subject.

The necessity of a stringent asepsis during a surgical procedure requiring between one and two hours for its completion is self-evident. The unusual difficulties encountered are peculiar to this operation and are purely of a mechanical nature: one is referable to the inaccessibility of the site of the operation—that portion of the portal vein extending about five to eight centimeters below the opening of the vena pancreatico-duodenalis, and the somewhat longer section of the vena cava between the

[1] Read before the American Association of Pathologists and Bacteriologists, April, 1904.

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hepatic and the renal veins; another is due to the extreme thinness and delicacy of the walls of the portal vein; but the chief difficulty is that of cutting an opening in the coapted walls of the two vessels within an oblong space bounded, except at one or two points, by a row of firmly tied sutures.

All who have successfully performed the operation have followed essentially the same technique up to the cutting of the fistula opening, except in the method devised by Queirolo (6) and later modified by von Karltreu (18, 22). Queirolo's operation has already been sufficiently criticised (7); von Karltreu's modification may correct the fault of Queirolo's method, but the distortion of the channels through which the blood must pass, and the traction upon the vena pancreatico-duodenalis are manifest disadvantages, and the operative results obtained by von Karltreu indicate that they are more than mere disadvantages. The question of the fact of the distance between the vena pancreatico-duodenalis and the first branchings of the portal vein into the liver[2] is one which every one can settle for himself. In my own experience it depends entirely upon the dog. The method described by Tansini (23) has not received the impress of experimental results; I do not believe it possible, because of the inaccessibility of the site of the operation.

The details of my operation are as follows. A dog—the size is immaterial—is anæsthetized by a mixture of one part chloroform to four parts ether; the abdomen is shaved and scrubbed and washed with 4 % carbolic acid; the dog and the operating table are then entirely covered with sterilized towels, the towel covering the dog's abdomen being made to adhere slightly by dampening with sterile salt solution. Towels, instruments, suture material, etc., are sterilized in the autoclave. An incision of sufficient length is then made in the median line, and the field of operation exposed by an assistant who holds the intestines toward the splenic side of the peritoneal cavity. The first step is the placing of the ligature around the portal vein below its first branchings into the liver and above the opening of the vena pancreatico-duodenalis, the tying of which, immediately

[2] See von Karltreu (22), p. 63.

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after the fistula opening has been made, will constitute the final step in the operation. The portal vein is then freed of the subperitoneal fat, if it be not already free for a sufficient extent, by blunt dissection.

An oval of the desired size, about three centimeters long by six to ten millimeters wide, is then mentally planned and a row of sutures is laid along the side of this oval nearer the spleen (Fig. 1). The sutures should be about three or four millimeters apart. The delicacy of the wall of the portal vein makes it necessary to pass the needles each time into the lumen of the vessel, a procedure which makes it essential that the needles be of extremely small size, and that the silk be of such nature as to fill completely the hole.

The needles which we have used are fashioned from the smallest size (No. 15) of round needle found on the market for use in bead work. They are made with a long eye, which greatly facilitates threading. The temper is drawn, the needles are cut to about two thirds their original length, sharpened to a very fine, round point, bent to a half curve, and then re-tempered. The needle-holder used is a Halstead mosquito hæmostat. The needles carry a fine, loose silk thread obtained by untwisting the two strands of the finest black embroidery silk; the resultant thread is composed of many filaments of silk, but is not twisted; used double, it fills completely the holes left by the needles; the needles are, however, so fine that the wall of the portal may be punctured by them with impunity. The wall of the vena cava is of such thickness that no difficulty is experienced even with larger needles. It is found most convenient to place all the sutures along one side of the oval before tying any of them, the two ends of each suture being caught with a hæmostat to prevent an entanglement of the various threads; after all are placed, the sutures are tied, the vessels being readily brought together by the act of tying the sutures.

The next step is to place in position in each vein the silk threads by means of which the cautery wire is later to be drawn into place (Fig. 1). The cautery wire consists of a fine platinum wire, made pliable by annealing, and provided with an eye at

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each end. Long silk threads of the same size as those used for the sutures are passed through each eye, and both ends of each thread then passed through the eye of a long, slightly curved needle. These needles must, because of their length, be larger than those used for placing the sutures, and the silk thread which they carry must be correspondingly thicker than that used for the sutures, in order to fill completely the holes made by the needles in the vessels. This is attained by passing both ends of the threads in the cautery wire through the eyes of the needles, and drawing them through for a sufficient distance to allow the needles, with the four-ply thread, to be drawn into and out of the vessels far enough to be out of the way of the subsequent steps (Figs. 2 and 3). One needle is inserted into the lumen of the portal vein at a point two or three millimeters below the end of the oval nearer the liver, and brought out of the portal at a point the same distance from the lower end of the oval; the other needle is introduced in the same manner into the vena cava (Fig. 1).

The oval is now completed by placing another series of sutures (Fig. 2), which are not tied until the next step in the operation—the drawing of the cautery wire into position by traction on the silk threads (Fig. 3)—has been performed. Since the cautery wire is of smaller diameter than the silk threads, some slight bleeding may occur at this time, but it soon stops, either by coagulation or after the next step in the operation—the tying of the sutures just laid (Fig. 4). The bleeding may be prevented entirely by tying the second series of sutures, except the one at either end of the oval, before drawing the cautery wire into place. We have usually, however, disregarded this hæmorrhage, which is rarely more than a mere oozing of blood, for the sake of having the assurance that the cautery wire is in its proper position. After tying the sutures of the second row, a suture is laid around the cautery wires (Figs. 3, 4, and 5) to close completely the lower end of the oval after cutting the opening. The threads are cut from the cautery wires, and the two ends of the wire threaded into the cautery (Fig. 5). The current is regulated by a simple water rheostat, which is set before the

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operation so that a platinum wire of the same size as the cautery wire is heated white-hot; it is usually found necessary to increase the strength of the current, which is done by an assistant, until the operator feels the yielding of the tissue due to the cutting by the cautery. As soon as the cautery wire frees itself from the tissues, the current is turned off, the cautery is removed, and the suture at the lower end of the oval is tied. The ligature laid around the portal as the first step in the operation is now tied; the operation is complete.

The abdominal wound is closed by a row of interrupted sutures through the *linea alba* and the peritoneum, and by interrupted sutures or a continuous suture of the skin. The wound is dressed with iodoform or bismuth-formic-iodide and covered with gauze dressing pads. The dog is then tightly bandaged, the bandage being covered with a muslin shirt—a piece of muslin of sufficient length to extend from the neck to the hind legs, provided with two holes through which the fore legs are passed, the ends being cut into narrow strips, which are tied along the middle of the dog's back. This bandage is left in place for a week or ten days. Bitches are chosen for the operation, because the male dog cannot be prevented from soiling the bandage with urine, and thus infecting the wound.

I mention these minor details because Pawlow writes as follows[3]: "A misfortune which has often occurred has pursued us during the whole course of our operations: six to seven days after a successful operation the edges of the wound separated. If this occurred during the night we usually found our animals dead in the morning. Sometimes we could help ourselves by immediately sewing the abdomen together again. It is to be hoped that later workers succeed in avoiding this truly annoying mishap by the use of another method of suturing."

I have not had such a misfortune in my short experience with the operation; indeed, the wounds have healed in nearly every case without even a stitch abscess. I have thought this result due to the use of a tight bandage rather than to the method of suturing.

[3] 3 (2), p. 167

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The construction of the cautery is shown in the diagram (Fig. 6). Two small German silver tubes (Fig. 6, a–b, a–b), insulated from each other, through which the cautery wire will easily pass, are soldered to insulated wires (Fig. 6, c, c). The insulation of the German silver tubes and the conducting wires is best accomplished by making the cautery of vulcanized fibre—the only material in our hands which has successfully withstood the heat transmitted from the platinum wire. The free ends of the cautery wire naturally remain cold; one end is held by the hand which holds the cautery in position, while traction with the other hand on the other end of the cautery wire draws the white-hot wire through the walls of the veins.

I believe that the use of the electro-cautery offers several distinct advantages over the best instrument hitherto used—Pawlow's modification of Eck's scissors. The points of these small scissors are soldered to long silver wires, the ends of which are soldered to curved needles. In regard to these scissors Pawlow himself says: "A certain number of animals succumbed because the walls of the veins were torn with the needles or with the wire ends of the scissors. Sometimes the operation did not succeed if the ends freed themselves from the blades of the scissors after the upper row [4] of sutures was already placed. In general the faulty cohesion of the soldered joint between the silver thread and the scissors is the weak side of our instrument, and it is only to be wished that one could find a better method of soldering, as well for the result of the operation as for the operator's peace of mind." [5]

In addition to avoiding this difficulty in the construction of Pawlow's scissors—which has been obviated, at least to a certain extent, by Italian workers, who substituted a copper wire for the silver wire—I believe the method above described offers at least two very distinct advantages. By the use of the soft silk threads in placing the cutting instrument in position, the danger of cutting the veins by a wire of any kind, during the considerable manipulation necessary for the placing of the second row of sutures, is entirely avoided. In the second place, the fact

[4] The second row.

[5] Loc. cit., p. 167.

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that the cutting instrument—the cautery wire—can be placed in actual position for cutting before the second row of sutures is tied permits the assurance that everything is in its proper position, and also makes it possible to tie all the sutures at the upper end of the oval before cutting the fistula opening. In Pawlow's method openings of sufficient size to permit the passage of the scissors must be left at both ends of the oval—openings through which a very considerable hæmorrhage may occur before the sutures placed ready for tying can be knotted. ("In the majority of cases the hæmorrhage is rather considerable, maximum sixty ccm." [6])

The use of the cautery makes it possible to close the upper end of the oval entirely, while the nose of the cautery occludes the lower end. The maximum hæmorrhage in our experience was about ten cubic centimeters, while ordinarily no hæmorrhage at all occurred. A further distinct advantage is that the cautery wire is so pliable that it can be introduced no matter how small the space is between the liver and the oval described by the sutures; while in Pawlow's method a space equivalent to the length of the scissors must be left between the lowest point of the liver and the highest point of the oval, since, whether the scissors be introduced from above or from below, they cannot be introduced or removed on a curve. The free portions of the veins are so limited, especially in small dogs, that any increase of available space is very welcome.

The question of the influence of the heat of the cautery upon the coagulation of the blood and the consequent formation of a thrombus in the fistula opening is one that I can answer only by referring to the results of my operations. The first six animals upon which the operation was performed, after the method described above was perfected, recovered perfectly and were utilized for further studies. Some of the results obtained have already been reported by Dr. P. B. Hawk, of the University of Pennsylvania (24).

The seventh dog, operated upon by Dr. H. J. Nichols, of Washington, D. C., at that time a fourth-year student in the

[6] Loc. cit., p. 166.

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University of Pennsylvania, recovered satisfactorily from the operation, but died three or four days later from an infection with an anaërobic, capsulated bacillus. Our operating-room had become infected with this micro-organism, and we lost six or seven other dogs from which the pancreas had been removed, from the same infection. An animal upon which I recently operated died during the following night for some reason which the autopsy did not satisfactorily reveal; the lungs showed extensive infiltration, but it was not determined whether the infiltration was to be ascribed to pneumonia or to embolism. The organs of the portal system showed no evidences of congestion. It is fair to state that the animal was in very poor condition before the operation was performed.

A ninth dog, recently operated upon, has recovered satisfactorily from the operation, making in a total of nine dogs, seven successful operations, one death from infection, and one death from what may have been the effects of the narcosis.

The work described above was performed at the Laboratory of Hygiene of the University of Pennsylvania, to the Director of which, Professor Abbott, I am indebted for every aid he could possibly extend me. I am further greatly indebted to my friend, Dr. C. R. Turner, Professor of Prosthetic Dentistry at the University of Pennsylvania, to whose patient teaching I owe the necessary knowledge of the working of metals. I would also express my thanks to Drs. Stewart, Hawk, Fager, Lewis, and Nichols, who acted as anæsthetists and assistants during the operations.

DESCRIPTION OF PLATES.

PLATE VI.

Fig. 1.—First row of sutures tied. Silk thread in place with which cautery wire is to be drawn into position.

Fig. 2.—Second row of sutures in position.

PLATE VII.

Fig. 3.—Cautery wire drawn into place.

Fig. 4.—Second row of sutures tied, except the safety suture around the cautery wire.

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PLATE VIII

Fig. 5.—Cautery wire threaded into cautery.

Fig. 6.—Construction of cautery. a–b, a–b, German silver tubes. c, c, wires insulated from each other.

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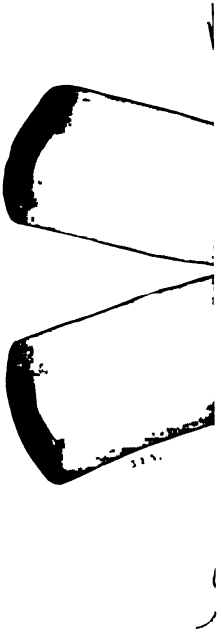

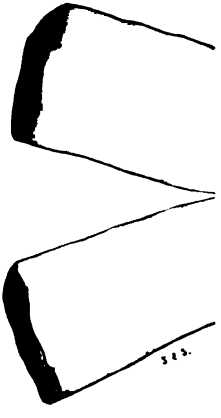

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corpuscles, from which he erroneously concluded that venom exerts its chief toxic action upon blood, and the various phenomena commonly observed in poisoned animals are merely the consequences of the loss of the respiratory function by the altered red corpuscles. Flexner and Noguchi [12] found the mechanism of venom hæmolysis to be analogous to serum hæmolysis, and demonstrated the occurrence in venom of hæmolytic amboceptors in Ehrlich's sense. Calmette [13] discovered the important fact, destined to have very far-reaching consequences, that one of the venom activating principles is thermostable, the other being of the nature of the usual serum complements. This observation of Calmette led immediately to the brilliant discovery of lecithin-venom activation by Kyes,[14] who also succeeded in preparing a crystalline compound of venom hæmolysin and lecithin which possesses all the native hæmolytic strength of the venom. The independence of the hæmatoxic and other toxic principles, the number of which is surprisingly large, has been shown by the later studies of Flexner and Noguchi.[15]

The properties of venom already enumerated depend on principles which, with the possible exception of the hæmolysin in the lecithin compound, have not been separated in a state approaching purity. Mitchell and Stewart [16] described another property of crotalus venom which had apparently escaped the attention of previous investigators. They observed that in a mixture of blood and fresh venom, in equal parts, the corpuscles, instead of undergoing hæmolysis, were actually preserved from disintegration for a period considerably greater than in the control specimens to which no venom had been added. On the other hand, if the amount of venom employed was less than 10 per cent of the mixture, hæmolysis occurred in the usual way. Mitchell and Stewart were led by their experiments to regard the once dried venom as being a less effective preservative than the fresh secretion, and they noted that, of the corpuscles tested, those of the rattlesnake were most perfectly protected by crotalus venom. Stephens and Myers [17] described a similar phenomenon in the case of cobra venom and human blood, although they do not state how long the corpuscles can be preserved in this way. Flexner

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and Noguchi [18] in their first studies noted a protection of rabbit corpuscles by means of rattlesnake venom, and, somewhat later, Kyes [19] and Kyes and Sachs [20] encountered the same phenomenon in the course of their studies with cobra venom, and Lamb [29] with daboia venom.

While the earlier investigators merely recorded their results, Kyes and Sachs [21] endeavored to explain the phenomenon. They accounted for it by supposing a complement (lecithin) deviation or "side-tracking" through the action of the large excess of venom hæmolytic amboceptors which were present in the mixture of blood and venom. Their crucial experiments were conducted as follows: Blood corpuscles were treated with a large excess of cobra venom and thoroughly washed to remove the excess of venom. Since, as Flexner and Noguchi [22] had shown, the venom amboceptors became attached to the corpuscles, the latter could now be hæmolyzed by contact with suitable complements (serum, lecithin). In the control experiments the excess of venom was not removed by washing, and in them the tubes containing the larger amounts of venom either failed entirely to show hæmolysis after addition of complement, or the amount of hæmolysis was greatly diminished.

In the course of a recent study of the influence of venom in promoting the hæmolysis caused by physical and chemical agents, this protective action of strong solutions of venom was given special attention, and the present communication deals with the results of this investigation. It may now be regarded as well established that when the optimum of the hæmolytic action of venom is exceeded, the degree of hæmolysis which it is capable of producing diminishes progressively as the dosage of the venom increases. Among the natural biological hæmolysins, venom alone is known to possess this property, but, in the course of a study of bacteriolysis by certain immune sera of high potency, Neisser and Wechsberg [23] observed an inhibition of bactericidal effects when an excess of amboceptors, relative to the complement content, was brought into the bacterial suspension. A similar although probably a distinct phenomenon has been described by Detre and Sellei [24] in their studies of hæmolysis caused by bichloride of

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mercury. But excepting the few experiments made by Kyes and Sachs, upon which they base their notion of complement deviation, no really extensive study of this property of venom has been made.

The explanation offered by Kyes and Sachs involves the belief that the principle, which is responsible for the protection, is identical with that which causes, under favorable conditions, dissolution of the corpuscles. If it can, therefore, be shown that it is not the hæmolysin which is protective, but some other principle, the notion that lecithin deviation explains the appearances can not be maintained. It is possible by means of suitably arranged experiments to test fully the assumption of a protective action exerted by venom and due to hæmolysin, and, further, to study other features of this action with a view of acquiring a more complete understanding of it.

The experiments to be related were devised to determine whether the protection is effective only against complements, or whether it operates as well against other hæmolytic agencies from which the biological factors are entirely eliminated. These several aspects of the subject will be dealt with in the following pages.

Preliminary Remarks upon the Action of Venom upon Blood Corpuscles.—In the experiments to be given, the blood of the horse was chiefly used, and it is meant unless another kind of blood is specified. Unwashed horse corpuscles are fairly susceptible to venom hæmolysis, while the corpuscles after washing (three washings in fifteen or twenty times their volume of salt solution at each operation) are insusceptible to venom. The horse has the advantage of yielding small or moderate quantities of blood at not too frequent intervals without causing perceptible changes in the resistance of the corpuscles. By using a single horse and establishing the resistance of its red corpuscles a standard can be obtained that, within small limits, is constant. In carrying out the experiments on resistance this condition was essential.*

*Theobald Smith (*The Journal of Medical Research*, 1904, xii, 385) has described a reduction in resistance to salt solutions of corpuscles of horses sub-

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The blood corpuscles of the horse present another advantage in their greater resistance to the mechanical injury involved in defibrination and washing.

The defibrinated blood in 0.9 per cent salt solution is susceptible to cobra, crotalus, and moccasin venoms, the varieties which were employed. . A mixture of blood suspension and cobra venom, in which the venom is contained in greater quantity than 5 per cent of the dry poison, will remain undissolved when kept for several hours at 20° C.; but at the end of twelve hours complete hæmolysis will have taken place.

In general it may be stated that rapidity and completeness of hæmolysis depend upon the proportional amounts of venom and blood in the mixtures: the greater the quantity of venom and the smaller the amount of blood, the slower and less perfect the hæmolysis. There is, however, one factor which controls to a large extent the rapidity and degree of hæmolysis, namely, the presence of serum constituents in the mixture. So long as a trace of serum remains, complete dissolution can not be prevented by any practicable concentration of venom. An equal mixture of defibrinated blood and 25 per cent cobra venom* solution will undergo hæmolysis in one or two hours.

It has already been stated that the thoroughly washed horse corpuscles are not hæmolyzed by venom no matter what the degree of concentration. If cobra venom be used in strengths above 4 per cent with a corpuscular suspension of 5 per cent, no change may take place in the mixture in many weeks; while with a quantity of venom as small as 0.1 per cent the corpuscles will disintegrate rather more quickly than in the control tubes which are not entirely sterile.

Corpuscles which had been brought into contact with the stronger solutions of venom were tested for resistance to salt solutions of varying tonicity. These tests disclosed the unexpected fact that corpuscles thus venomized, and in the

jected to repeated bleedings in the production of diphtheria antitoxin. Our practice was to take relatively small quantities of blood at intervals of a number of days from a strong horse that had not previously been used for any experiment.

*The percentage of dried venom is always meant.

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presence of an excess of venom, are not hæmolyzable even by water.

At the same time their susceptibility to heat is changed. It has been found that control tubes of blood corpuscles alone are hæmolyzed completely in from one hundred and seventy-five to one hundred and eighty minutes when kept at the constant temperature of 53° C. In the presence of venom of a concentration not exceeding 1 per cent, complete hæmolysis will take place at this temperature in from five to fifteen minutes; with a concentration of venom as low as 0.01 per cent thirty minutes will be required; with a concentration of 10 per cent there is no perceptible change in the corpuscles for the first twenty minutes, after which laking commences. This last laking is not, however, typical. A bright hæmoglobin color does not appear in the fluid, but the cells undergo disintegration, the color of the mixture becomes coffee-like, and in about one hour a turbid precipitate of cell particles and venom granules can be made out under the microscope.

Strong solutions of venom are capable of protecting the corpuscles from destruction by water, but venom solutions below 2 per cent in strength render the corpuscles more sensitive to salt solutions, as measured by the tonicity of the fluid for corpuscles; and as the strength of the venom falls from this limit the susceptibility, as measured by the degree of tonicity, diminishes.

Is the Protective Action Due to Venom Hæmolysin?—The first question to be considered is whether the protective action of strong solutions of venom is in any way dependent on the hæmolytic amboceptors. Since this protection is exhibited against water and heat, it is safe to assume that it bears no necessary relation to complement deviation. However, even these reactions do not prove that the hæmolysin is unconcerned in the process. Some other explanation, one, namely, in which an alteration in solubility of the protoplasmic constituents of the cells is concerned, has more likelihood of fitting the facts observed, but nevertheless it is necessary to exclude by suitable means any possible participation of the hæmolysin.

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There is a marked difference between the effects produced by complements present, in an available form, before the venom has acted on the corpuscles and those produced by complements added after the treatment with venom. Should even a trifling quantity of serum be left with the corpuscles, it is not practicable to obtain the protective effect of venom, while if the effect is once produced and the excess of venom allowed to remain, very large amounts of serum or of lecithin fail to bring about hæmolysis.

The first set of experiments relates to the difference in effect produced by two concentrations of venom. In each experiment the washed corpuscles were in 5 per cent suspension, and were allowed to remain in contact with the venom for two hours before any complement was added. The mixtures were all made in 0.9 per cent solution of salt. The two strengths of venom were 10 and 2 per cent. Table I expresses briefly the result.

TABLE I

5 per cent horse corpuscles.	Amount of corpuscular suspension. c.c.	Amount of lecithin and fresh serum. c.c.	Result, at 20° C
10 % Cobra venom	0.3	+ 0.9 % salt sol. 0.7	= No hæmolysis
	"	+ $\frac{1}{1000}$ N. lecithin 0.7	= No hæmolysis
	"	+ $\frac{1}{100}$ N. " 0.7	= Doubtful result, owing to the formation of granules, but no hæmolysis after 6 hours.
	"	+ horse serum 0.7	= No hæmolysis; strong discoloration after some hours.
2 % Cobra venom	0.3	+ 0.9% salt sol. 0.7	= No hæmolysis.
	"	+ $\frac{1}{1000}$ N. lecithin 0.7	= Complete hæmolysis in 5 minutes.
	"	+ horse serum 0.7	= Complete hæmolysis in 2 hours.
	"	+ horse serum 0.07	= Complete hæmolysis in 4 hours.
Control 0.9 % Salt sol.; no venom	0.3	+ 0.9 % salt sol. 0.7	= No hæmolysis.
	"	+ $\frac{1}{1000}$ N. lecithin 0.7	= Complete hæmolysis in 8 hours.
	"	+ $\frac{1}{100}$ N. " 0.7	= Complete hæmolysis in 1 hour.
	"	+ horse serum 0.7	= No hæmolysis.

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Examination of the table shows that the corpuscles which are acted on by 10 per cent venom are not hæmolyzable by quantities of fresh horse serum and lecithin, which far surpass the amounts theoretically required to bring about solution; while with the venom in 2 per cent solution the corpuscles were perfectly dissolved by lecithin and serum. The quantity of complements used in the experiments given in the table was in great excess, and the excess in the tests with 10 per cent venom was ten times greater than in the tests with 2 per cent venom, and one hundred times greater than actually required under optimal conditions.

Before discussing the participation of the venom-hæmolysin in the protective action, it will be necessary to describe the influence of heat upon the reaction. As a preliminary step, the influence of high temperatures upon the hæmolytic action of cobra venom was made. It was found that the filtrate from the coagulum of venom heated for five minutes at 100° C. was still hæmolytic, and, indeed, quantitatively in the same degree as the unheated venom.

Tubes containing a 25 per cent solution of cobra venom in 0.9 per cent salt solution were heated to 95°, 100°, and 135° C., respectively. The temperatures of the first two tubes were maintained for five minutes, while the last tube was kept at the high temperature in the autoclave for one hour. When venom in solution is heated to 95° C. it becomes milky in appearance, while at 100° C. coarse particles of coagulated matter separate. At the temperature of 135° C. the coagulum shrinks to a solid, spongy mass, leaving the fluid portion clear. The heated venom was now tested and it was found that the specimen heated to 95° C. was practically unaltered in its hæmolytic and protective action; while at 100° C. it had lost a portion of its effect only, and at 135° C. it had lost entirely both of these properties.

The next series of tests was made with the separated portions, coagulum and filtrate. For this purpose the specimen heated to 95° C. was employed, and to obtain a perfectly clear fluid, filtration through a Berkefeld bougie was resorted to. The results of this test were conclusive; the filtrate had no protective action,

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while the coagulum when suspended again was protective. The filtrate from the specimen heated to 100° C. was also non-protective. The filtrates from both of these fractions contain the hæmolytic principle in undiminished amounts.

These experiments prove definitely that the protective principle is distinct from the hæmolytic amboceptors, and it is interesting to note that the coagulum from the specimen heated to 95° C. is still capable of exerting protective influence upon blood corpuscles. It seems safe to state that the coagulated proteid is not the protecting body; and hence it is a fair assumption that in process of coagulation the protective principle is mechanically carried down under conditions of union which still permit its entering into combination with blood cells.*

Although the filtrate from a solution of venom heated to 95° or 100° C. has no protective action, such a filtrate exerts an injurious action on the corpuscles, whereby they are rendered more easily hæmolyzed by salt solution. The washed corpuscles of the horse are laked by 0.44 per cent salt solution, but after treatment with the filtrates mentioned they are instantly laked by solutions of 0.5 per cent. The temperature of 135° C. destroys not only the protective but also the injurious body; and at that temperature the filtrate is without hæmolytic action even in the presence of suitable complements.

Our experiments lead us, therefore, to regard corpuscular protection by strong solutions of venom as a peculiar effect which is opposed to hæmolytic action, and one which has thus far received neither adequate attention nor probable explanation. Moreover, it would seem that the hæmolyzing principle in venom is directly injurious to corpuscles even in cases in which suitable complements are absent. Until a special injurious agent which predisposes corpuscles to laking by physical agents is discovered in venom, we are inclined to believe that the injurious action of the filtrates is due to venom hæmolysin.

* In view of the fact that in his experiments which led to the view of complement deviation by venom amboceptors Kyes employed rabbit corpuscles, I have made a series of tests with these cells. My results show that rabbit corpuscles are somewhat less perfectly protected than some other cells, but

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A passing consideration should be given to venom agglutinins, since it is at least conceivable that the agglutinated masses of corpuscles arising from venom treatment are less readily affected by laking agents than the separated corpuscles. But since the

they exhibit unmistakable evidences of protection to heat, saponin, and water. Moreover, they also show the paradoxical reaction to salt solution, since the super-venomized corpuscles show at one point a greatly lessened resistance to solutions of sodium chloride. A table is appended in which these results may be observed.

Amount of 5 % cobra venom solution c. c.	1 c. c. of 5 % suspension of rabbit blood (washed)		
	Blood No. I.		Blood No. II.
	Temperature of 53° C.	1 % saponin sol.	Reaction to water and NaCl solution.
1	No hæmolysis, precipitate formed, cells disintegrated in two hours.	Slight hæmolysis	Slight hæmolysis in water.
0.5	No hæmolysis, precipitate formed, cells disintegrated in two hours.	" "	Partial hæmolysis in water.
0.25	Complete hæmolysis in 9 minutes	Partial "	Complete hæmolysis in 0.5 % salt sol.
0.1	" " 5 "	Complete "	" " 0.53 % " "
0.075	" " 3½ "	" "	" " 0.68 % " "
0.05	" " 2 "	" "	" " 0.566 % " "
0.025	" " 2½ "	" "	" " 0.55 % " "
0.01	" " 9 "	" "	" " 0.535 % " "
0.005	" " 17½ "	" "	" " 0.52 % " "
0.001	" " 35 "	" "	" " 0.48 % " "
0.0005	" " 42 "	" "	" " 0.458 % " "
Control (no venom)	" " 130 "	" "	" " 0.43 % " "

In repeating Kyes's experiments I found that the points of protection coincided with the points of so-called deviation of complement as is shown in the next table.

Amount of 5 % cobra venom solution c. c.	1 c. c. of 5 % rabbit blood (defibrinated)		
	Blood No. I.	Blood No. II.	Blood No. III.
1	No hæmolysis	No hæmolysis	No hæmolysis
0.5	" "	Trace "	" "
0.25	Trace "	Marked "	Slight "
0.1	Moderate hæmolysis	" "	Moderate hæmolysis
0.075	Almost complete hæmolysis	Complete hæmolysis	Complete "
0.05	Complete hæmolysis	" "	" "
0.025	" "	" "	Almost complete hæmolysis
0.01	" "	Almost complete hæmolysis	" "
0.005	" "	" "	Marked hæmolysis
0.001	Almost complete hæmolysis	Much hæmolysis	" "
0.0005	Trace hæmolysis	No "	No "
Control (no venom)	No "	" "	" "

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venom agglutinins are destroyed at relatively low temperatures their participations can be quickly eliminated. I have also tested abrin and ricin, which are strongly hæmagglutinative, without finding a corresponding protection. Indeed, corpuscles treated with abrin are slightly reduced in resistance, while corpuscles treated with ricin show about the normal reaction to salt solution.

The serum of the rattlesnake is highly agglutinative and hæmolytic for corpuscles of the horse, and yet it does not protect them in any degree. The corpuscles, after a contact of twelve hours with the inactivated rattlesnake serum in excess, were hæmolyzed by salt solution of 0.45 per cent strength.

That other coagulated proteids and mucin do not contain any such protecting principle is shown by the negative results obtained with coagulated serum of the horse and of the rattlesnake, and with salivary mucin.

The Effect of Washing the Corpuscles after Venomization.—Attention has been drawn to the fact that the property of venom which is being described is operative only in the presence of an excess of venom. Corpuscles once protected can be diluted with water indefinitely without any laking taking place, so long as the excess of venom is not removed by washing, and other evidences of protection, such as increased resistance of the corpuscles to heat and to various chemical laking agents, can be demonstrated. If, however, the venomized corpuscles are subjected to repeated and thorough washing with 0.9 per cent salt solution, it is found that the protection disappears and, moreover, the corpuscles are now more easily laked than normal blood cells.

Four mixtures of washed corpuscles and venom were prepared. The corpuscles were used in 3 per cent suspension in salt solution, and the venom strengths were 10, 1, 0.1, and 0.01 per cent. The period of contact was six hours. A portion of each mixture was removed and washed in 0.9 per cent saline four times, twenty times the volume being used at each washing. Two sets of tubes, after dilution four times with saline solution, were exposed to the temperature of 53° C. and the results noted. They are reproduced in Table II.

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TABLE II

Strength of venom solution.	Washed venomised corpuscles.	Unwashed venomised corpuscles.
10 %	Complete hæmolysis in 20 minutes	No typical laking, but general disintegration of cells in 60 minutes; the fluid presented a light brownish hue.
1 %	Complete hæmolysis in 25 minutes	Complete hæmolysis in 6 minutes.
0.1 %	Complete hæmolysis in 25 minutes	Complete hæmolysis in 12 minutes.
0.01 %	Complete hæmolysis in 30 minutes	Complete hæmolysis in 30 minutes.
Control (no venom)	Complete hæmolysis in 170 minutes	Complete hæmolysis in 175 minutes.

Table II shows that horse corpuscles suspended in the venom solutions of strengths above 0.1 per cent absorb about the same quantity of venom irrespective of the actual concentration of the solutions. The quantity of venom absorbed sufficed to bring about laking in twenty-five minutes, the mixtures having been kept during this period at 53° C.; and this amount of venom is apparently the maximum quantity which the corpuscles are able to retain after repeated washings with the saline solution.

There is no method of ascertaining with absolute accuracy whether this quantity was originally absorbed, or whether it is the part of the original quantity fixed to cells and retained with great avidity. An approximate estimation could, of course, be made of the amount of venom remaining in the fluid part of the mixture after simple contact of the blood-cells and venom; but since, in carrying out this estimation, no account can be taken of the venom which is loosely and mechanically held by the corpuscles, figures approaching accuracy are impossible.

That venom directly injures blood corpuscles has been mentioned, but Table II is valuable in showing that this injury can be expressed in quite definite figures. I know of no series of experiments in which laking by means of heat has been used to

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determine the degree of decreased resistance of blood cells. The method seems to promise a fair degree of accuracy, and it also would seem to promise the discovery of substances of primary injurious nature, which until now have been assumed to be inactive except in the presence of suitable complements.

In order that this primary injurious action may be expressed, the concentration of the venom solution must be kept between 0.1 and 1 per cent. Higher concentrations either begin to be protective or do not increase the injury, because they are not so entirely absorbed. In any case an excess of venom would appear not to be present in the unheated mixtures, for hastening of heat hæmolysis is not observed in the venomized corpuscles which have previously been washed. Hence the question arises whether at the high temperature more venom is not absorbed than at 20° C.; from this increased fixation and injury perhaps the greater susceptibility to laking proceeds. No answer to this question is attempted at present; but I shall now proceed to the next series of experiments which are concerned with the effect of repeated washings in saline solution upon the tonicity of the venomized corpuscles, as measured by their resistance to salt solutions of different strengths.

TABLE III

Strength of venom solution.	Venom removed.	Venom not removed.
10 %	0.51 %	Not hæmolyzable in water.
1 %	0.49 %	0.48 %
0.1 %	0.48 %	0.465 %
0.01 %	0.465 %	0.44 %
Control (no venom)	0.44 %	0.44 %

The striking difference in the manner of reaction to hypotonic solutions between the corpuscles acted upon by strong venom solutions and by solutions of weaker concentration is well illustrated in this table. After the action of 10 per cent venom, the corpuscles can be diluted indefinitely with water without showing any tendency to dissolve, while the same corpuscles, after

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repeated washing with 0.9 per cent salt solution, show a higher hypotonic index than normal—0.51 per cent as compared with 0.44 per cent. The significance of this behavior is quite plain. Strong venom solutions react in some manner with the corpuscles so that they are rendered insoluble in water; the insoluble compound is, however, capable of being broken up by certain chemicals—in this case sodium chloride. But whether it is the sodium chloride as such, or the free ions in the mixture, the experiment does not show. It may be remarked here that the protective property is not quickly removed by the saline solutions, but several washings are required to remove it. It would seem, therefore, that an excess of sodium chloride alone does not suffice, but some other factor is concerned.

Since it has now been shown that the entire protective effect of the venom, as regards water hæmolysis, can be removed by washing the corpuscles with the saline solution, the question arises whether this process restores the susceptibility of the corpuscles to lecithin and serum hæmolysis. It has already been stated that the presence of serum in the mixture will prevent the strongest solutions of venom from causing protection; and now it may be said that the protection once removed by salt solution the corpuscles become susceptible to serum and lecithin hæmolysis.

The next table gives the results obtained with lecithin and horse serum. The conditions of the experiment were similar to those of the preceding experiments. The period of contact of venom and washed corpuscles was two hours; and the washing was always done with 0.9 per cent salt solution. The volume of suspended corpuscles was 3 cubic centimeters, to which 0.5 cubic centimeter of $\frac{1}{1000}$ N. lecithin or 1 cubic centimeter of horse serum was added. The observations in the case of the horse serum were made at the end of four hours.

It appears that washing with salt solution exposes the protected corpuscles to laking with serum and lecithin. This susceptibility to laking is due to the fixation of venom hæmolysin by the corpuscles. When the activity of very strong solutions, after removal of all unattached venom, is compared with the

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activity of weak solutions, in which subsequent washing is omitted, a marked difference in the rapidity with which hæmolysis occurs is apparent. The difference may depend, possibly on one of two causes, first, that the corpuscles in the absence of complement actually fix an insufficient quantity of hæmolysin to produce rapid laking; or, next, the washing removes a part of the hæmolytic amboceptors already attached.

The Action of Certain Substances upon the Venomized Blood Corpuscles.—The effects of water, salt solution of different concentrations, and heat upon the venomized corpuscles have been

TABLE IV

Strength of venom solution.	LECITHIN.		HORSE SERUM.	
	Venom removed.	Venom not removed.	Venom removed.	Venom not removed.
10 %	Complete hæmolysis in 28 minutes	No hæmolysis	Complete hæmolysis	No hæmolysis.
1 %	Complete hæmolysis in 28 minutes.	Complete hæmolysis in 7 minutes	" "	Complete hæmolysis.
0.1 %	Complete hæmolysis in 29 minutes.	Complete hæmolysis in 9 minutes.	Partial hæmolysis	" "
0.01 %	Complete hæmolysis in 30 minutes.	Complete hæmolysis in 28 minutes.	" "	" "
Control (no venom)	Complete hæmolysis in 8 hours.		No hæmolysis	

recorded. The next series of experiments deals with the action of still other definite agents, such as acids, alkalies, etc., upon the protected cells. The method of procedure was as follows: Four tubes, containing 4 cubic centimeters each of 5 per cent cobra venom in 0.9 per cent salt solution and 6 per cent of washed horse corpuscles, were allowed to stand for two hours at 20° C. Tube No. 1 received 4 cubic centimeters of water, and tube No. 2 received 20 cubic centimeters of water; the corpuscles were permitted to subside when the clear fluid was decanted and the volume of the residue brought up to 8 cubic centimeters. Tube No. 3 received an additional 4 cubic centimeters of salt solution, while the residue in tube No. 4 was washed once in 20 cubic centimeters of salt solution and suspended in 8 cubic centimeters of saline solution. The actual tests were made by measuring 2

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cubic centimeters of each mixture into a test-tube, to which the solutions of chemicals were added gradually until an amount was introduced which produced instantaneous hæmolysis. The chemicals were dissolved either in water or in saline solution, according as they were to be added to blood cells diluted in one or the other of these fluids. The results are given in the next table.

TABLE V

Venomized corpuscles.	$\frac{1}{10}$ N. HCl c. c.	$\frac{1}{10}$ N.NaOH c. c.	$\frac{1}{10}$ N. NH_3 c. c.	0.1 % saponin. c. c.
1. Dilution with water	0.125	0.75	1.75	No hæmolysis.
2. Sedimented and resuspended in water	0.12	0.7	1.	" "
3. Dilution with saline solution	0.33	1.5	2.	" "
4. Washed with and resuspended in saline solution	0.25	0.8	0.3	Complete hæmolysis, 0.35
5. Control, no venom	0.5	1.75	0.4	" " 0.45

An analysis of this table shows an essential agreement between the manner of action of hydrochloric acid and sodium hydroxide. These substances cause hæmolysis in smaller quantities when added to the watery suspensions than with suspensions of corpuscles in saline solution, the difference being approximately as 1 : 2 or 1 : 2.5. On the other hand, after removal of the excess of venom by washing in the saline solution, the corpuscles show a greatly reduced resistance, since they are about twice as readily laked by these chemicals as the control without venom. On the other hand, in the watery medium the super-venomized corpuscles are about four times as susceptible to hydrochloric acid and twice as susceptible to sodium hydrate as the control specimens. This apparent paradox is probably explained by the nature of the medium, for, the insoluble compound of venom and proteid once dissolved, the previously protected corpuscles

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immediately become the victims of water-laking. The reduction of resistance exhibited by the venomized cells suspended in salt solution is an expression of the injury inflicted by the venom directly upon the corpuscles in the manner already described.

On the other hand, ammonia permits unmistakable evidences of protection to be exhibited. In the first place, in the presence of excess of venom, ammonia proved to be relatively a weak solvent for the protecting compound, and, in the next place, in the case of the washed venomized cells the susceptibility of the corpuscles to the solvent action of the ammonia is increased beyond the control, although this difference is again less marked than with hydrochloric acid and sodium hydrate.

Another remarkable fact is the inability of saponin to cause laking of the protected corpuscles. According to Kobert,[25] saponin is hæmolytic probably because of its property of dissolving lecithin, which it withdraws from the corpuscles with the effect of destroying the integrity of the cells. It would seem, therefore, that the protection of the corpuscles against saponin hæmolysis might be associated with a change in the availability of the intracellular lecithin. It is improbable that venom extracts the lecithin, for if it did it is likely that the corpuscles would be directly disintegrated by the venom, but such disintegration does not occur. It is far more probable that the venom action is to bring about some physical or chemical alteration in the cells which prevents the usual effect of saponin upon the corpuscles. That, indeed, the effect of venom is not permanent, as would be the case were it to abstract the lecithin, is shown by the results of washing the protected corpuscles in the saline solution and subsequent exposure to saponin. Such washed cells are hæmolyzed by saponin even more readily than the controls. Hence the evidence is in favor of the view that venom fixation of the corpuscles is due to some change in the character of the cellular constituents, and that the venom acts also directly upon the corpuscles, producing an injury even though this injury is not at once evident. We shall return to the first aspect of this subject. The strongest solutions of venom which it is practicable

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to use, like the weaker solutions, act injuriously upon blood corpuscles. The stronger solutions, however, exert a second action —a kind of fixation of the cells—which obscures the injurious action. Table VI gives the results obtained with saponin and corpuscles in the presence of an excess of venom and after its removal. The volume of the venomized blood suspension in each instance was 4 cubic centimeters. Saponin was employed in 0.1 per cent solution, which was added in increasing quantities until instantaneous hæmolysis was produced.

TABLE VI

Strength of venom solution.	Washed venomized corpuscles + 0.1 % saponin.	Unwashed venomized corpuscles + 0.1 % saponin.
10 %	0.3 c. c.	No hæmolysis *
1 %	0.35 "	0.35 c. c.
0.1 %	0.4 "	0.4 "
0.01 %	0.45 "	0.45 "
Control (no venom)	0.45 "	0.45 "

Strong venom solutions afford no protection against formic acid hæmolysis, and the protection against oleic acid is not absolute, but the degree of resistance against this acid was not wholly worked out. Ether in large quantities fails to lake the venom-protected corpuscles.

The Rapidity and the Limits of the Protective Action.—In order to determine the rapidity of the fixation of the corpuscles by venom a series of tests was made. A 10 per cent solution of cobra venom was prepared in 0.9 per cent salt solution, and to this washed horse corpuscles were added in quantity of 3 per cent. The mixture was allowed to stand at 20° C. for the period of the experiment, and at the intervals mentioned in the table portions were withdrawn and tested against salt solutions of different degrees of hypotonicity. A parallel series of tests was carried on to determine what concentration of venom in 0.9 per cent saline solution is necessary to protect the corpuscles against suspension in water. In the latter series a contact of five hours

* 2 % saponin in the mixture caused no hæmolysis after 10 days.

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was employed. The following table presents the results of these experiments:

TABLE VII

DETERMINATION OF RAPIDITY OF ACTION.		DETERMINATION OF MINIMAL LIMIT OF ACTION.	
Period of contact of venom and corpuscles.	Reaction to sodium chloride solution.	Concentration of venom solution.	Reaction to sodium chloride solution.
5 minutes	Partial hæmolysis in 0.3 % sol.	2 %	Complete hæmolysis in 0.7 % sol.
10 "	Trace hæmolysis in 0.3 % sol.	3 %	Trace hæmolysis in 0.1 % sol.
20 "	No hæmolysis in 0.3 % sol.; trace in water.	4 %	No hæmolysis in water.
30 "	No hæmolysis in water.	5 %	No "
60 "	" " "	6 %	No "
		7 %	No "
		8 %	No "
		Control (no venom)	Complete hæmolysis in 0.44 % sol.

Estimation of the Protective Action of Moccasin and Crotalus Venom.—The tests under this heading were made in the manner previously given. Different strengths of the venoms were prepared in 0.9 per cent saline, and to them washed horse corpuscles were added in 3 per cent suspension. The mixtures were allowed to stand for six hours at 20° C., and were then tested against salt solutions of varying tonicity. The removal of the venom in some of the experiments was done by means of 0.9 per cent saline.

TABLE VIII

Concentrations of venom.	CROTALUS VENOM.		WATER-MOCCASIN VENOM.	
	Venom removed. Tonicity of NaCl sol.	Venom not removed. Tonicity of NaCl sol.	Venom removed Tonicity of NaCl sol.	Venom not removed. Tonicity of NaCl sol.
20 %	0.54 %	0.18 %	0.57 %	0.2 %
10 %	0.528 %	0.2 %	0.48 %	0.25 %
5 %	0.5 %	0.3 %	0.47 %	0.35 %
1 %	0.49 %	0.465 %	0.47 %	0.465 %
0.1 %	0.47 %	0.454 %	0.465 %	0.45 %
0.01 %	0.454 %	0.444 %	0.45 %	0.445 %
Control (no venom)	0.44 %		0.44 %	

The above table brings out the interesting fact that, while moccasin and rattlesnake venoms possess the property of

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protecting blood corpuscles, they are in this respect much inferior to cobra venom. With neither moccasin nor rattlesnake venom was there produced absolute protection against water hæmolysis. It may be worth while to point out in this place that in ultimate hæmolytic power with defibrinated horse and dog corpuscles these venoms were about the equal of cobra venom, although crotalus venom acts more slowly and is quantitatively a little weaker than the others.* The fact of the similarity in hæmolytic power and dissimilarity in protective action is another proof of the distinction between the several principles causing the two phenomena.

TABLE IX

Period of contact before heating.	Time required for complete hæmolysis at 53° C.
Immediate	10 minutes.
10 minutes	11 "
30 "	20 "
60 "	38 "
2 hours	30 "
6 "	25 "

The Influence of Small Amounts of Venom upon Heat Hæmolysis.—In the course of the experiments a phenomenon came under my observation which will now be described. Mixtures of washed corpuscles and venom of low concentration were exposed, after varying intervals of contact, to the temperature of 53° C. At first the effect, as measured by the degree of heat hæmolysis, is small, but after a time it becomes marked. However, after a still longer interval of contact the acquired resistance undergoes diminution. A series of tests is given in Table IX. The mixture exposed to heat consisted of 20 per cent washed horse corpuscles

* For complete hæmolysis under the conditions mentioned, crotalus venom requires at 20° C. twenty-four hours and, in addition, occasional agitation of the tubes. If the comparison of hæmolysis be made after six hours and at the upper limit of dosage, the difference between cobra and moccasin venom, on the one hand, and rattlesnake venom, on the other, may be as 1 : 20, but if the reading be taken after twenty-four hours it will be as low as 2 : 3 or, at most, 2 : 4.

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in 30 cubic centimeters of 0.9 per cent salt solution, and 0.2 per cent cobra venom in 1.5 cubic centimeters of the same strength salt solution. Four cubic centimeters were removed at a time at different intervals and tested.

We can see in these results the opposed action of the two properties of venom when acting together. The first effect to declare itself is apparently partial fixation of the corpuscles, but when the venom solution is too weak to accomplish this entirely, the directly injurious action of the poison makes itself felt. Hence it would seem that with such weak solutions of cobra venom as were employed in this experiment, the injurious action upon the corpuscles, in the absence of available complement, was more marked than the protective action.

The Action of Venom upon the Blood Serum, the Stroma, and the Soluble Contents of the Blood Corpuscles.—With a view of ascertaining whether venom forms with the blood constituents any difficultly soluble compound, it was tested separately upon the fluid and solid portions. Horse blood being used, the serum, the stroma, and the corpuscular contents soluble in water were tested against various venom solutions.

The stroma was prepared by laking the twice-washed, condensed corpuscles. The final product was a gelatinous mass of pale rose color, which could be suspended in water or in saline solution. The contents of the corpuscles were readily obtained by laking with water and centrifuging. They could also be diluted to any degree with water or salt solution.

The results given in the table show clearly that venom is a precipitant for certain of the constituents of the blood. The chief precipitate is obtained with the water-soluble contents of the corpuscles, although a partial reaction is also obtained with diluted serum. The stroma reacts in a minor degree with the venom, but whether or not this reaction depends on the venom agglutinin or is due to a precipitant in the real sense was not determined.

The precipitate formed with the contents of the corpuscles in pure water is dense, viscid, and finely divided, while in saline solution it is coarser and less in amount. The original color of

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TABLE X

Cobra venom sol.	10 % SERUM.		10 % STROMA.		10 % CORPUSCLE-CONTENTS *		CONTROL VENOM SOLUTIONS.	
	in water.	in 0.9 % salt sol.	in water.	in 0.9 % salt sol.	in water.	in 0.9 % salt sol.	in water.	in 0.9 % salt sol.
5 %	Partial precipitation.	Cloudy, but evenly so.	Cloudy, but not more than the control.	Cloudy, but not more than the control.	Dense, viscid, and soft precipitate formed quickly. The precipitate separates out, leaving clear, slightly tinted fluid near the surface.	A coarser precipitate is formed after 18 hours. By diluting with a large volume of water a more abundant and finer precipitate appears.	Cloudy, but evenly so.	Cloudy, but not quite opaque.
1 %	A definite precipitation.	Slightly cloudy.	Slightly cloudy; the stroma settled down to the bottom.	Slightly cloudy; the stroma settled down to the bottom.	Similar to the above; but precipitate much less opaque.	A definite precipitation, but less than with water. Dilution with water caused increase of precipitate.	Slightly cloudy.	Slightly cloudy.
0.1 %	A slight cloudiness.	Almost no cloudiness.	Almost clear; the stroma sunken to the bottom.	Almost no cloudiness; the stroma is on the bottom.	More cloudy than the control, but no definite precipitate.	About same as the control.	Almost no cloudiness.	Almost no cloudiness.
No venom (control)	Almost no cloudiness.	Almost no cloudiness.	Perfectly clear; the stroma is all sedimented.	Perfectly clear; the stroma is all sedimented.	Perfectly clear.	Perfectly clear.		

* Contained hæmoglobin also.

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the solution is bright red from the presence of dissolved oxyhæmoglobin, but venom quickly changes it to dark brown. The pigment is carried down with the precipitate in the watery mixture, but in the saline mixture it remains for some time in the supernatant fluid. Salt, in certain concentrations would seem, therefore, to prevent, more or less, the formation of the precipitate. Indeed, if the saline solution described is further diluted with water a more abundant precipitation takes place.

Since the protected corpuscles are quickly dissolved by acids and alkalis, the question naturally arises whether the precipitate is also easily soluble in these chemicals. It was found that acids and alkalis dissolve the precipitate in approximately the same degree as they are capable of bringing about hæmolysis of the venom-treated corpuscles.

The Action of Venom upon the Separated Constituents of Serum and Corpuscles.—Venom, as has been stated, causes a dense precipitate with an aqueous extract of washed corpuscles. When, however, sodium chloride is present in the solution in a strength equalling or exceeding 0.9 per cent, the precipitation does not take place, or occurs only imperfectly. Now, it is my belief that venom owes the protective action upon corpuscles described to its ability to form a compound insoluble in water with certain corpuscular constituents. It is, therefore, desirable to ascertain whether any particular constituent of the corpuscles is concerned in the formation of the insoluble substance, or whether all the proteids of the cells are fixed by the venom. Moreover, since salt in weak concentration is able to prevent the development of the precipitate in the corpuscular extract, it is also possible that it prevents, in serum, a similar reaction. The next experiments were made with serums in which the saline percentages had been reduced by dilution with water. The results of these tests are given in Table XI.

That simple dilution with water affects the precipitability of a compound formed by venom and serum when brought together, is shown by the results given in the table. Another way of approaching this problem is to remove the salts of the serum by dialysis. This was done for the serum of the horse and dog, and

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dialysis was continued until a considerable amount of globulin had separated from the serums. It was found that only a trace of chlorides remained in the fluid. The two parts—fluid and precipitated globulin—were tested separately. The globulin which had separated belonged chiefly to the euglobulin fraction, but there still remained in the fluid parts of the serums considerable globulin precipitable by one-third saturation with ammonium sulphate. During the dialysis the volume of the serums had increased two and one-half times.

TABLE XI

	MIXTURE A.	MIXTURE B.	MIXTURE C.	MIXTURE D.
	Undiluted serum 1.0 c. c. 20 % cobra venom 0 2 c. c.	Mixture A 0.2 c. c. Water 1.0 c. c.	Serum diluted with 4 vols. of water 1.0 c.c 20% cobra venom 0.2 c. c.	Serum diluted with 4 vols. of 0.9 % salt solution 1.0 c. c. 20 % cobra venom 0.2 c. c.
Horse serum	Clear	Moderate precipitate	Much precipitate	Clear
Rabbit "	"	Moderate "	Moderate "	"
Rat "	"	Much "	Much "	"
Guinea-pig "	"	" "	" "	"
Dog "	"	" "	" "	"
Pigeon "	"	" "	" "	"
Crotalus plasma	"	Much precipitate, resembling fibrin.	" "	"

REMARKS: Controls without venom remained perfectly clear for more than 24 hours, while the controls with venom alone showed only a slight amount of precipitate in water and in 0.9 % salt solution.

The dialyzed horse serum was tested as follows: First tube—1 cubic centimeter of the fluid portion was mixed with 0.2 cubic centimeters of 20 per cent cobra venom solution; moderate precipitation. Second tube—The same mixture plus sodium chloride to 0.9 per cent strength; no precipitation. Next, the precipitate of globulin was washed in water by centrifuging and dissolved or suspended in salt solutions of the following concentrations: 0.8, 0.6, and 0.4 per cent. In the first there was perfect, in the second almost perfect, in the third imperfect solution. To the clear filtrates the 20 per cent venom solution was added, and rapid and nearly complete precipitation of the dissolved globulins occurred. When the globulin is merely suspended, as in water or weak saline, the venom quickly brings down the sus-

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pended particles which, left to themselves, subside very slowly, if at all. A comparison of the amounts of sediment obtained from the fluid part of the dialyzed serum and the redissolved globulin shows that the globulin is more completely precipitable than the other constituents.

It was now decided to fraction the serum, and for the purpose the method of Freund and Joachim [26] was employed. The fractions—euglobulin, pseudoglobulin, serum albumin—were freed from ammonium sulphate by dialysis and tested with cobra venom. The most marked reaction was given by the first, the pseudoglobulin fraction giving a less reaction, and the serum-albumin fraction no precipitate. The results of the two series of tests, therefore, agree in showing the globulin to be the constituent acted upon by the venom.

The water-soluble contents of the corpuscles were also fractioned. The first fraction, yielded by one-third saturation, was proportionately large and contained a part of the coloring matter of the corpuscles. The second fraction, obtained by one-half saturation, was less in amount than the first, but contained the greater part of the oxyhæmoglobin. The third fraction, obtained by saturation, was small in quantity. The salts were removed by dialysis.

The first two fractions were deep red in color. One cubic centimeter of the solution of each was mixed with 0.2 cubic centimeters of 20 per cent cobra venom; the result was precipitation. The second fraction gave the more abundant precipitate, leaving the fluid clear of coloring matter. In order to obtain similar clearing of the first fraction additional dilution with water was required. The third fraction yielded no precipitate with the venom solution.

Since in this series of experiments the globulins and hæmoglobin were associated and it was desirable to know whether the reaction takes place between hæmoglobin and venom, as well as between the globulins and venom, separation of the hæmoglobin in a pure state was necessary.

Oxyhæmoglobin from horse corpuscles was prepared by Zinoffsky's [27] method, while that from dog's corpuscles was prepared by

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laking the washed corpuscles with ether and removing the stroma in the separatory funnel. The clear solution of hæmoglobin was shaken with a large volume of ether and allowed to stand for twenty-four hours at 0° C. A large quantity of crystalline oxyhæmoglobin, which was purified by being twice recrystallized, was thus obtained.

For testing against venom the hæmoglobin was used in 0.2 to 0.1 per cent solutions in water or 0.9 per cent sodium chloride. To 1.0 cubic centimeter of the solutions of oxyhæmoglobin 0.1 cubic centimeter 20 per cent venom was added. The results were as follows: in the presence of sodium chloride no reaction occurs; in aqueous solution there is complete precipitation of the hæmoglobin of the horse, but no reaction with that of the dog. That pure hæmoglobin may react with venom is shown by these experiments; that the reaction is similar to that with the globulins in respect to solubility in salt is also shown; and that a difference in composition exists between dog's and horse's oxyhæmoglobin the experiments also indicate.

In view of these results it seemed desirable to test venom directly upon globin, which was prepared from the oxyhæmoglobin of the horse and dog by Schulz's [28] method. The solutions of globin were neutral to litmus and gave the reactions described by Schulz. To 1.0 cubic centimeter of globin solution 0.1 cubic centimeter of 20 per cent venom was added. Dog's globin gave no precipitate; horse's globin gave a voluminous precipitate which was easily soluble in acids and alkalis, but not in salt solution. Indeed, a small quantity of salt hastens the appearance of the precipitate with horse's globin.

This series of experiments would tend to show that the precipitability of oxyhæmoglobin by venom depends on the globin constituent, and that the globins of horse and dog corpuscles are distinct substances as regards, at least, their reaction with venom.

The question has already been mooted as to what the nature of the protective process described in this paper really is. That the phenomenon is independent of the hæmolytic reaction has been conclusively proven. Moreover, the immediately preceding experiments show that venom is a precipitant for several proteids

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of the blood. It is doubtless upon the reaction which takes place between the venom and these precipitable proteids that the phenomenon of protection depends. One of the striking appearances of the reaction is the carrying down of the hæmoglobin by the venom; and it seems not improbable that this property might be utilized as a measure of the protection of the corpuscles, for when it is absent it is found that venom does not shield the corpuscles from water laking. The following table shows that this relation actually exists:

TABLE XII.

Origin of corpuscles.	20 % cobra-venom solution 2.0 c. c. Washed blood corpuscles 0.3 c. c.		1 % intracorpuscular contents 1.0 c. c. 20 % cobra-venom solution 0.1 c. c.	
	Behavior of the corpuscles left in the above mixture.	Behavior of the corpuscles when brought into water.	Precipitation of the hæmoglobin.	Solubility of the precipitate* in 0.9 % sod. chloride solution.
Horse	No hæmolysis	No hæmolysis	Complete	Dissolves but reprecipitates on standing.
Rabbit	" "	" "	Almost complete; fluid retains a faint color.	Partially soluble; large precipitate on standing.
Rat	" "	" "	Complete	Slightly soluble
Guinea-pig	" "	" "	None	" "
Dog	Complete hæmolysis	Complete hæmolysis	"	
Pigeon	Slight "	" "	Slight	Easily soluble.
Crotalus	No "	Incomplete "	"	" "

* The precipitate was easily soluble in different acids and alkalis.

Yet, since it is not only the hæmoglobin which is precipitated by venom, but the intracorpuscular globulins as well, it is not possible to describe the phenomenon of protection as if it depended wholly on a reaction with the former proteid. It is probable that all the precipitable proteids are acted upon and affect the result, although it seems not unlikely that the fixation of the hæmoglobin in an insoluble form is what directly prevents its diffusion out of the corpuscles into the surrounding medium.

In the course of this paper allusions to a change in the permeability of the corpuscles have been made. The question as to whether the so-called protected corpuscles are altered in their physical condition so as to exclude certain substances, which under normal conditions easily enter them, is pertinent. It is

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to be considered whether the rendering of certain of the proteids insoluble is not associated with an obstruction to osmotic processes taking place between the corpuscles and the surrounding medium. If in the case of water and the protected corpuscles this fluid was successfully excluded from the corpuscles by an insoluble although superficial barrier, laking could not occur. However, many of the experiments described show that the absence of laking does not depend upon changed conditions of osmosis, but upon the formation of insoluble compounds.

A set of experiments was undertaken to determine whether, at the same time, the permeability of the corpuscles may not have been altered. For this purpose saponin was employed, since, as stated, Kobert has shown that saponin causes hæmolysis by virtue of the fact that it dissolves the lecithin of the corpuscles. Now, the failure of saponin to act upon the protected corpuscles could be due either to their impenetrability for saponin, or to the insolubility of the venom-hæmoglobin compound in the menstruum in spite of the change of the cells caused by a solution of the lecithin in saponin. The following experiment was devised with a view of determining the nature of the protection from saponin.

Twenty per cent cobra venom in salt solution was treated with washed horse corpuscles (5 cubic centimeters of venom plus 0.5 cubic centimeters of corpuscles) for three hours at the room temperature. The corpuscles were rendered water-protected and also non-hæmolyzable when treated with 2 per cent watery solution of saponin.

Two cubic centimeters of the venomized corpuscles were dropped into 150 cubic centimeters of distilled water; sedimentation was complete in twenty hours. The corpuscles were twice washed in the centrifuge with water. Examined microscopically they were entangled with venom granules. These corpuscles were protected perfectly both from water and 0.9 per cent salt solution. The sediment was mixed with an equal volume of 2 per cent solution of saponin, and the mixture was allowed to stand for ten minutes. The corpuscles were diluted and washed in water until all the saponin (determined by testing

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with unprotected corpuscles) was removed. A thick suspension in water of the saponin-venomized corpuscles was now added separately to water and to 0.9 per cent saline solution (0.2 cubic centimeter of suspension to 2 cubic centimeters). The salt solution quickly caused laking, while, as was to be predicted, the water was without effect.

The interpretation of this experiment is probably as follows: The saponin enters the venom-protected corpuscles in spite of their fixation and takes up the lecithin, whereby the corpuscles are seriously damaged. However, as the venom-hæmoglobin compound is insoluble in water, no visible expression of this damage can occur. Since the venom-hæmoglobin compound is soluble in 0.9 per cent saline solution, it quickly dissolves in the latter whether it is capable of leaving the corpuscles or not. Unless the corpuscles have been previously injured, the dissolved hæmoglobin compound does not diffuse into the medium; but in view of the action of the saponin upon the corpuscles a sufficient injury has been inflicted to allow the hæmoglobin to escape. Venom protection of blood corpuscles does not seriously affect the permeability of the corpuscles, but it prevents, under many conditions, the diffusion of the hæmoglobin into the surrounding medium.

SUMMARY.

Washed blood corpuscles of certain species of animals in a concentration of about 5 per cent suspended in salt solution containing above 4 per cent of cobra venom undergo changes in their resistance to certain physical and chemical agents. They become non-hæmolyzable by water, ether, saponin, and quite strong solutions of lecithin, provided always that the excess of venom has not been entirely removed. On the other hand, certain acids and alkalis, excepting ammonia, lake the venomized corpuscles more easily than they lake normal corpuscles. Venom solutions of 2 per cent and less exert no protective property upon blood corpuscles, but they induce changes in the corpuscles whereby they are rendered more easily laked by the same physical and chemical agents.

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The changes in the corpuscles upon which this protection relies can be set aside by repeated washings of the venomized corpuscles in 0.9 per cent salt solution. When the protection is removed the corpuscles are left in a state of diminished resistance to injurious agencies.

The substance in venom upon which the protective action depends is not destroyed by the temperature of 95° C., although at this temperature a part of the venom is coagulated. The protective body is found in the coagulated portion; while the venom hæmolysin is contained, in full activity, in the clear filtrate. The agglutinin for red corpuscles disappears at 75° C.; hence, the protection of corpuscles by venom depends neither upon the hæmolysin nor the agglutinin.

Blood corpuscles absorb the hæmolysin from concentrated solutions of venom as readily as from weaker solutions. This fixed hæmolysin can be brought into action, under suitable conditions, by the addition of complements. But the hæmolysin is unable, in the presence of complements in excess, to cause laking of the corpuscles unless the protection produced by the venom has first been set aside.

Cobra venom forms a precipitate with blood serum when comparatively free of salts or when diluted with water; it forms a precipitate with a watery extract of blood corpuscles, and with the separated globulins, hæmoglobin and globin of the blood. The precipitates are insoluble in water, but dissolve in weak acids and alkalis. The stroma of the red corpuscles causes no change in venom solutions.

Protection of the corpuscles against hæmolysis depends upon the formation by venom of a water-insoluble compound with certain of the corpuscular constituents, chiefly probably with hæmoglobin. There is no reason to believe that the permeability of the corpuscles is greatly altered.

The restoration of the susceptibility of the corpuscles to the ordinary laking influences is accomplished by dissolving the venom-proteid compound. Since this compound is soluble in acids and alkalis and even in a large excess of salt solution, estoration is easily effected. Corpuscles which have been pro-

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tected and again rendered hæmolyzable are less resistant to injurious influences than normal corpuscles, resembling in this respect corpuscles which have been treated with relatively weaker solutions of venom. Venom, therefore, always exerts a direct injurious influence upon blood corpuscles; but strong solutions of venom may obscure this injurious effect through the predominance of the protective action which they display.

Not all blood corpuscles are susceptible to the protective action of venom, and different corpuscles display varying degrees of capacity for protection. Dog corpuscles are not protected by cobra venom, and watery extracts of dog corpuscles, as well as pure oxyhæmoglobin and globin of this animal, are not precipitated by venom solutions.

Crotalus and moccasin venoms possess this protective property, but in less degree than cobra venom.

I wish to express my gratitude to Professor Flexner for his encouragement and for many valuable suggestions during the execution of this work.

ADDENDA.

After the completion of this study the paper of Sacharoff and Sachs (*Münchener med. Wochenschrift*, 1905, lii., 297) on the hæmolytic action of photodynamic substances came to my attention. I therefore tested the protected corpuscles of the rabbit by exposing them directly to sunlight in the presence of eosin. It was found that the protected corpuscles are not subject to the hæmolytic action of this florescent substance; while the corpuscles which have had the protection removed by means of salt solution are readily hæmolyzed by eosin when exposed to the sun's rays. In keeping with the other experiments upon the injurious effects of smaller and non-protective doses of venom, it was also found that venomized but unprotected corpuscles are more readily hæmolyzed by eosin in the light than the normal corpuscles.

Another fact may be added. The supervenomized corpuscles have become insusceptible to tetanolysin.

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Darstellung und Analyse einiger Nucleinsäuren.

XI. Mitteilung.

Über die Nucleinsäure der Kuhmilchdrüse.

Von

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(Der Redaktion zugegangen am 18. August 1905.)

Über die Natur der in der Milchdrüse enthaltenen Nucleinkörper liegen nur wenige Arbeiten vor. Die Untersuchung von Odenius[1]) richtete sich auf die Analyse des Nucleoproteids und führte zu der Annahme, daß der fragliche Körper in seinem Molekül eine Pentose, und nur eine Purinbase — das Guanin enthält. Die Arbeit von Basch[2]) schien den Beweis dafür geliefert zu haben, daß die Nucleinsäure, die aus der Drüse dargestellt werden kann, die Zusammensetzung einer Paranucleinsäure besitzt. Nach der Ansicht dieses Forschers soll sich das Casein der Milch in der Weise bilden, daß die freie Paranucleinsäure der Milchdrüsenzellen sich mit dem Serumeiweiß zu einer komplizierten Substanz vereinigt.

Die vorliegende Untersuchung beschäftigt sich mit der Zusammensetzung dieser Nucleinsäure, und zwar hat sich dabei ergeben, daß ihre Bestandteile sich von denen der echten Nucleinsäuren nicht unterscheiden.

Die Substanz wurde nach dem Verfahren dargestellt, welches der Eine[3]) von uns kürzlich angegeben hat. Die Drüsen wurden durch Waschen mit kaltem Wasser möglichst vollständig von Milch befreit, zerhackt und mit salzhaltigem Wasser ge-

[1]) Läkaref. Farm. (N. F.), Bd. V, und Malys Jahresber., Bd. XXX.
[2]) Jahrb. f. Kinderheilkunde, 1898, S. 88.
[3]) Diese Zeitschrift, Bd. XLV, S. 370.

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kocht. Darauf wurde die Säure durch Behandlung mit Alkali extrahiert, durch Essigsäure und Pikrinsäure von Eiweißsubstanzen befreit und dann mit Alkohol gefällt.

Nach dem angegebenen Verfahren gelangte man zu biuretfreien Substanzen.

Zur Analyse wurde das Kupfersalz der Säure benutzt. Nachdem es im Toluolbad zwei Stunden getrocknet war, zeigte es die folgende Zusammensetzung:

Präparat I.

0,2150 g Substanz gaben 0,2480 g CO_2 und 0,0788 g H_2O; C = 31,40%, H = 4,07%.

0,1560 g Substanz gaben 0,1790 g CO_2 und 0,0575 g H_2O; C = 31,28%, H = 4,08%.

Das aus 0,5479 g Substanz nach Kjeldahl erhaltene NH_3 neutralisierte 48,6 ccm H_2SO_4 (1 ccm = 0,00162 g N), d. i. N = 14,37%.

0,1432 g Substanz gaben 0,0432 g $Mg_2P_2O_7$; P = 8.49%

0,1859 » » enthielten 0,0500 g Asche = 26,88%.

Präparat II.

Das aus 0,1204 g erhaltene Ammoniak neutralisierte 11,1 ccm H_2SO_4 (1 ccm = 0,00162 g N), d. i. N = 14,93%.

0,18972 g Substanz gaben 0,0566 g $Mg_2P_2O_7$; P = 8,42%

0,1538 » » enthielten 0,0402 g Asche = 26,13%.

Die folgende Tabelle zeigt die Zusammensetzung der Substanz verglichen mit der in gleicher Weise aus Milz dargestellten Nucleinsäure.

Aus Milz: C 34,94, H 4,57, N 15,30, P 8,15, Cu 7,5 %.

» Milchdrüse: » 31,34, » 4,07, » 14,65, » 8,48, » 7,00%.

Die zwei Präparate unterscheiden sich also nur in dem Gehalt an Kohlenstoff und Wasserstoff. Man darf aber nicht außer acht lassen, daß das Salz mit Alkohol entwässert war, und daß man nicht feststellen kann, wie viel von diesem auch nach dem Trocknen an dem Salz haften blieb.

Purinbasen.

35 g lufttrockner Substanz mit einem Phosphorgehalt von 7,35% wurden im Autoklaven mit 200 ccm 2%iger Schwefelsäure zwei Stunden erhitzt. Das Produkt der Hydrolyse wurde filtriert, das Filtrat mit Barytwasser alkalisch gemacht, vom

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Niederschlage abfiltriert, der Überschuß von Baryum mit Schwefelsäure entfernt und die Flüssigkeit nach Zusatz von Ammoniak eingedampft. Der dabei gebildete Niederschlag wurde abfiltriert, in wenig Alkali aufgelöst und mit Essigsäure neutralisiert. Es bildete sich dabei ein Niederschlag, der als Guanin zu betrachten war. Es betrug 0,300 g. Für die Analyse wurde er in das Sulfat übergeführt.

Das aus 0,1407 g der lufttrockenen Substanz nach Kjeldahl erhaltene NH_3 neutralisierte 36,4 ccm Schwefelsäure (1 ccm = 0,00124 g N).

Berechnet für $(C_5H_5N_5O)_2\,H_2SO_4 + 2H_2O$:	Gefunden:
N = 32,11 %	32,07 %

Aus dem Filtrate wurde das Adeninpikrat in üblicher Weise dargestellt. Die Ausbeute betrug 1,3 g. Zur Reinigung wurde die Substanz in kochendem Wasser bei Zusatz von möglichst wenig Alkali gelöst, die Lösung mit Salzsäure genau neutralisiert und das Adeninpikrat abfiltriert, ohne die Flüssigkeit erkalten zu lassen. Im Toluolbade getrocknet, zeigte die Substanz den Schmelzpunkt 279° C.

Das Filtrat vom Adeninpikrat wurde von Pikrinsäure durch Schwefelsäure und Toluol und Äther befreit, und mit ammoniakalischer Lösung von Silberchlorid behandelt. Es bildete sich dabei ein ganz geringer Niederschlag, der sich nicht zur weiteren Analyse eignete.

Pyrimidinbasen.

25 g des Kupfersalzes wurden im Autoklaven mit 75 ccm 25 %iger H_2SO_4 drei Stunden auf 150—175° C. erhitzt, das Reaktionsprodukt mit Äther extrahiert, sodann mit heißem Wasser verdünnt; die überschüssige Schwefelsäure mit Barytwasser entfernt und das Filtrat von Baryumsulfat auf ein kleines Volumen eingedampft. Es schied sich dabei ein Niederschlag ab, der hauptsächlich aus Thymin bestand. Einmal aus 2 %iger Schwefelsäure umkristallisiert, betrug die Ausbeute 1 g Thymin.

Das aus 0,156 g der Substanz nach Kjeldahl erhaltene NH_3 neutralisierte 21,1 ccm Schwefelsäure (1 ccm = 0,00162 g N).

Berechnet für $C_5H_6N_2O_2$:	Gefunden:
N = 22,22 %	21,91 %

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Die Filtrate vom Thymin wurden mit Barytwasser von Schwefelsäure befreit und mit konzentrierter Pikrinsäurelösung versetzt. Der nach 24 Stunden gebildete Niederschlag wurde als Cytosinpikrat in Rechnung gezogen. Er betrug 2 g.

Das Pikrat wurde sodann in das basische Sulfat übergeführt. Die Kristalle hatten das typische Aussehen dieses Salzes. Es wurde dann in Chloroplatinat umgewandelt.

0,1240 g dieses Platinsalzes gaben 0,0388 g Pt.

Berechnet für $2(C_4H_5N_3O) \cdot PtCl_4 \cdot 2HCl$:	Gefunden:
Pt = 30,84 %	31,29 %

In der folgenden Tabelle sind Ausbeuten der Basen für 100 g Nucleinsäure berechnet. Daneben sind die Mengen der in gleicher Weise aus Milznucleinsäure erhaltenen Basen angeführt.

	Milznucleinsäure	Nucleinsäure aus Milchdrüse
Adeninpikrat	8,27 g	4,56 g
Guanin	1,62 »	1,05 »
Thymin	5,71 »	5 »
Cytosinpikrat	21,43 »	10 »

Kohlehydrate.

Die freie Säure gibt mit Orcinsalzsäure eine violette Färbung und der amylalkoholische Auszug zeigte die für Pentosen typischen Absorptionsstreifen. Bei Anwesenheit geringer Spuren von Kupfer erhält man bei derselben Behandlung eine tief grüne Färbung. Die Absorptionsstreifen bleiben unverändert. Bei der Destillation mit Salzsäure konnte Furfurol im Destillate durch essigsaures Anilin und Phloroglucin nachgewiesen werden.

Aus der nach Spaltung der Säure mit konzentrierter Schwefelsäure resultierenden Flüssigkeit konnte mit Äther Lävulinsäure extrahiert werden. Aus den für die Pyrimidinbasenanalyse angewandten 25 g der Substanz wurde eine ganz erhebliche Menge des Silbersalzes der Lävulinsäure dargestellt.

Für die Analyse wurde die Substanz einmal aus heißem Wasser umkristallisiert.

0,1640 g des Salzes gaben 0,0795 g Ag.

Berechnet für $C_5H_7O_3Ag$:	Gefunden:
Ag = 48,43 %	48,47 %

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Reprinted from The Journal of Experimental Medicine, 1905.

A STUDY OF THE CHANGES IN THE BLOOD AND BLOOD-FORMING ORGANS PRODUCED BY CYTOTOXIC SERA, WITH SPECIAL REFERENCE TO HÆMOLYMPHOTOXIN.[1]

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PLATES IV AND V.

Since this article was originally planned, many changes have been made in its scope and general outline, not only because the great amount of study devoted to the cytotoxins has rendered some of the work first planned unnecessary, but also because the opinions concerning the cytotoxins, their mode of action, specificity, etc., have undergone many changes. Perhaps the most radical change of ideas is in regard to specificity, many observers having bent their energies towards definitely proving or disproving the specificity of the cytotoxins. The one who has perhaps done most towards proving the non-specificity of the cytotoxins is Pearce, who, in a recent paper before the American Association of Pathologists and Bacteriologists, has clearly demonstrated that the changes which have been regarded as specific by most writers are due to hæmolysins and agglutinins; when these factors, the former in particular, are considered, the changes cannot be regarded as specific,—they are not changes in kind, but are rather changes in degree alone.

In the investigations here reported, an attempt is made to compare several cytotoxins to find what changes they produce, and, if the changes are merely in degree, to determine to what extent these changes occur after the injection of different cytotoxins, and the order in which the degree of the change would

[1] Aided by a grant from the Rockefeller Institute for Medical Research.

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place them. Furthermore, an attempt is here made to produce a hæmolymphocytotoxin and by its aid to throw if possible some light upon the physiological action of the hæmolymph glands. So far no attempt has been made to produce a hæmolymphocytotoxin and to study the effects produced by it; in doing so an entirely new field is entered.

Taken for granted that the hæmolymph nodes are organs *sui generis*, as demonstrated by Warthin (1), what then is their true function? Some of the theories may be stated as follows:

1. They are hæmolytic, and therefore destructive organs, and rid the body of old and partially broken-down red blood cells.

2. They are centres for the formation of leucocytes and blood plasma, and in these respects conservative.

3. They are centres for the production of large numbers of eosinophile cells; the granules arising from the disintegration products of red cells are taken up by mononuclear leucocytes, the nuclei of which become polymorphous.

4. They compensate in some degree for the spleen in case of inability of that organ to perform its proper function.

5. They are centres for the destruction of leucocytes.

6. Since they are enlarged in certain infections, they play a part in the production of immunity and in the protection of the body through the formation of anti-bodies.

The suggestion by Warthin and Lewis (2) of a hæmolytic ferment produced by these glands brings forth more strongly the possibility that they play an important part in the production of certain forms of anæmia. In apparent confirmation of this suggestion, Warthin, in eight autopsies on cases of pernicious anæmia, has found the hæmolymph nodes exceedingly red and hyperplastic and showing signs of over-activity. One object of this paper, then, is to prove if possible the presence of such a ferment; for, if it is present, a more hæmolytic serum should be produced by the injection of hæmolymph nodes than would be produced by injection of defibrinated blood, diluted to a corresponding percentage of red blood cells.

Throughout this work it will be frequently necessary to refer to the effects of lymphotoxin and myelotoxin on the blood and

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blood-forming organs. The literature of these subjects has of late become so extensive that a review of it here is impossible; some of its aspects, however, have been most excellently reviewed in the articles by Flexner (3) and by Bunting (4) to whose work references will often be made.

METHODS AND MATERIALS.

The basis of the present investigation is that a specific toxin for a tissue may be formed by the injection of that tissue into an intermediary animal. Glands were obtained from sheep and injected into geese; the serum of the goose, after a sufficient time had elapsed for attaining its highest toxicity, was injected into a sheep, the tissues of which were then examined for evidences of a specific toxin.

In producing the toxin geese and sheep were used, the former on account of their ability to withstand repeated injections without becoming infected, and the latter because the hæmolymph glands obtained from them are larger and more numerous than from the animals ordinarily used for experimental purposes.

The geese received suspensions of glands prepared by the method in general use. Glands removed under as nearly sterile precautions as possible were first triturated in a sterile mortar, suspended in sterile physiological salt solution, and allowed to extract for twelve hours; they were strained through sterile gauze and injected into geese. The injections were made intraperitoneally at intervals of from three to ten days until seven or eight injections had been made; at the end of this time the geese were bled.

When the geese were injected with blood, blood was first defibrinated and then diluted with physiological salt solution in such quantity that the percentage of red blood cells was the same as that contained in a corresponding injection of hæmolymph nodes, so that the results might be comparable.

The geese used were as follows:
Goose 2 furnished normal serum.
Goose 5 furnished normal serum.

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Goose 3, 4, 6, and 12 each received seven injections of hæmolymph glands, extending over a period of about thirty-six days.

Goose 7 and 14 received seven injections of defibrinated blood diluted as above.

Goose 11 received 6 injections of lymph glands.

Goose 15 received 8 injections of endothelium.

Goose 16 received 5 injections of liver.

Goose 17 received 5 injections of kidney.

Goose 18 received 5 injections of spleen.

(Note.—Goose 1 and Sheep 1 were used in a preliminary experiment performed a year ago, but not included in the present series of experiments.)

NORMAL HISTOLOGY OF THE SHEEP.

The tissues to be examined were removed from normal sheep, fixed in formalin and mercuric chloride, and stained with hæmotoxylin and eosin. These methods were uniformly applied to the pathological organs. Various special stains were also employed when deemed necessary. For the iron reaction, potassium ferrocyanide and hydrochloric acid were used.

Blood.—Counts of the total number of leucocytes as well as differential counts were made shortly before injection of the sheep and at varying intervals for four or five days after. At the end of this time the sheep were killed and sections made from all the organs. In counting the cells of the blood, the Thoma-Zeiss apparatus was used and at the same time smears were made for differential counts, the latter being fixed in alcohol and ether and stained with methylene blue and eosin.

The average leucocyte count of a sheep is about 8000, but varies between the wide limits of from 6000 to 12,000 per cubic millimetre. The erythrocyte count may also vary greatly and still be within normal limits, but averages eight million per cubic millimetre.

In order that the results might be comparable, the following cells were taken into consideration in making the differential counts, this classification of leucocytes being used by Bunting in his work on the blood of the rabbit:

1.—Mononuclear Leucocytes,
    a—Lymphocytes,
    b—Large Mononuclears,

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2.—Polymorphonuclear Leucocytes,
a—Pseudo-eosinophiles (Amphophiles),
b—Eosinophiles (coarsely granular),
c—Basophiles (Mast Cells).

The last are so few in numbers in the sheep that they might well be left entirely out of consideration. The average percentage and limits of variation as obtained from about twenty counts from normal sheep may be given as follows:

Lymphocytes.....	53 %	40 to 60 %
Large Mononuclears....	8 %	3 to 11 %
Amphophiles.....	37 %	30 to 55 %
Eosinophiles.....	1.7 %	.2 to 8 %
Mast Cells.....	.3 %	0 to 2 %

It is readily seen from the above that the different forms may vary within rather wide limits and still be normal.

Hæmolymph Glands.—The hæmolymph glands of the sheep are typical in structure, the majority being of the splenolymph type. They have a thick capsule from which trabeculæ, forming either a delicate framework or a coarse framework densely infiltrated with lymphoid tissue, extend into the gland, dividing it into irregular lobules. The lymphadenoid tissue, while varying greatly in amount, is usually abundant and forms the greater part of the gland. As a rule, large numbers of nodes with germ centres showing fairly active proliferation are present, although in a few cases cords alone are found. The peripheral blood sinus is generally large but may be frequently interrupted by lymphadenoid tissue projecting into it. The gland shows a few phagocytes, some pyknotic and vesicular nuclei, and a light sprinkling of eosinophiles, the latter being for the most part polymorphonuclear, although a few mononuclear forms are to be found.

Lymph Glands.—The lymph glands are almost identical with human glands in structure, having a thick cortical substance composed of lymph nodules and germ centres, and a medullary substance composed of anastamosing cords separated by trabeculæ. The entire gland is surrounded by a rather thick capsule.

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Only a few eosinophile cells are seen and these are chiefly around the borders of the lymph nodes.

Spleen.—The spleen has a thick capsule, and the trabeculæ throughout the organ are conspicuous. The Malpighian bodies are large, distinct, and not very numerous. The lymphoid cells in the pulp are scanty, the reticulum being relatively increased, and the reticular cells large; the organ seems to show an over-abundance of connective tissue.

Bone Marrow.—The bone marrow cells found in the sheep having practically the same characteristics as those of the rabbit, the following classification as given by Muir (5) for the latter animal may here be used:

1.—Leucocytic class of cells:

a—Cells with finely granular protoplasm which constitute the great majority of the cells in the marrow. After mitotic division they become the finely granular amphophile leucocytes of the blood.

b—Cells with coarse eosinophile granules which later become the ordinary eosinophiles of the blood.

c—Hyaline or non-granular cells.

Small lymphoid cells.

Large lymphoid cells.

Large mononuclear cells.

2.—Erythrocytes.

3.—Erythroblasts:

a—Megaloblasts or large nucleated red corpuscles faintly tinted with hæmoglobin; these are the youngest variety of erythroblasts.

b—Normoblasts or typical nucleated red corpuscles, which are somewhat larger than red blood cells and are the oldest variety of erythroblasts.

4.—Giant Cells.

5.—Connective-tissue class of cells, fat cells, etc.

EXPERIMENT WITH PHYSIOLOGICAL SALT SOLUTION.

As noted by Besredka (6), Ricketts (7), Brinckerhoff and Tyzzer (8), and later by Bunting, injections of fluids intra-

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peritoneally cause first a leucopenia and later a hyperleucocytosis of the peritoneal fluid. It is probable that within certain limits the greater the toxicity of the fluid injected the greater the resulting hyperleucocytosis. (Ricketts.) The bone marrow and lymph glands in attempting to compensate for this withdrawal of leucocytes from the circulation give rise to a rather typical blood picture. Bunting found after the injection of cold sterile physiological salt solution a slight rise in the total leucocyte count of the circulating blood. On differential analysis this increase was found to be due to a marked amphophile leucocytosis, at the same time that there was a correspondingly great lymphopenia. The preliminary leucocytosis he calls the "anticipatory" leucocytosis, and is due to the reaction of the bone marrow to the withdrawal of leucocytes from the circulation, the lymphocytes having a slower amœboid movement failing to respond so quickly.

As these experiments were performed on rabbits it was thought best to repeat them with sheep so that the influence of salt solution might be exactly determined for that animal and could then be eliminated from the final results. After the injection of thirty-five cubic centimetres of cold sterile physiological salt solution (Table I),* a rather sharp leucocytosis and a return to normal within twenty-four hours is obtained, and is followed by another lesser rise and fall in the next forty-eight hours. By differential counts we find at first a typical "anticipatory" leucocytosis with its accompanying lymphopenia, the lymphocytes, however, recovering quite rapidly. The second leucocytosis is due to an increase both of the amphophiles and of the lymphocytes with a final rather gradual return of both to normal.

The bone marrow, then, in the sheep as well as in the rabbit, reacts quickly to a demand upon it, the lymphocytes, because of their slower amœboid movement, responding more slowly.

INJECTION OF NORMAL SERUM OF THE GOOSE.

In the following experiments no consideration has been taken of the amount of hæmolysis *in vitro*, this being reserved for a

* The tables are given at the end of the article.

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future study. The blood curve (Table II) resulting from the injection of normal serum is almost identical with that obtained by Bunting, showing a marked leucocytosis at the end of eighteen hours, followed by a fall, preceding a second and finally a third slighter rise. On differential analysis the first rise is found to be due almost entirely to an amphophile leucocytosis, except for a sharp rise of lymphocytes, with a sharper return to normal, occurring within eight hours. This preliminary increase in lymphocytes is in marked contrast, however, to the observations of Bunting, who found a typical "anticipatory" leucocytosis, as described, with its accompanying lymphopenia. The second and third rises are due entirely to an increase in lymphocytes; the number of amphophiles decrease and subsequently there is only a slight secondary rise to normal.

The large mononuclear leucocytes increase in numbers following roughly the rise and fall of the lymphocytes; the eosinophiles, after a preliminary sharp decrease in numbers, gradually rise considerably above normal and then decrease; the erythrocytes show a slight increase at first and subsequently their number remains elevated; the basophiles are so few in the sheep that they have not been enumerated in the table.

SHEEP 2.—Received 35 cc. of serum from Goose 5 at 3 P.M., December 1, 1903. A record of the leucocyte counts and differential analysis is given in Table II. The animal was killed December 8, 10 A.M. *Autopsy.*—There is slight œdema near the site of inoculation. A large amount of fluid is present in the peritoneal cavity. The hæmolymph glands are exceedingly large and numerous. All the organs and tissue are apparently normal. There is no infection.

Histology.—The structures examined showed little or no changes other than a slight hyperplasia. This is perhaps most noticeable in the bone marrow, as here the actual amount of cellular marrow is increased, the increase corresponding to a diminution in the number of fat cells. The change is due to an increase in number of the marrow cells or myelocytes. The megaloblasts and normoblasts are also more numerous than usual and show mitotic figures. The germ centres in the spleen and lymph glands stand out distinctly and show active although not excessive proliferation. Few, if any, degenerative changes are present.

The injection of normal serum of a goose into a sheep causes first a diminution in the number of the leucocytes in the circulating blood as a result of their withdrawal into the peritoneal

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cavity. This fall is followed by a marked rise, due to proliferation of the antecedents of the leucocytes, first and most markedly in the bone marrow and later in the lymph glands, spleen, and hæmolymph glands. At the same time there is a slight increase in number of erythrocytes in the blood due to a proliferation of the erythroblasts in the bone marrow. The changes produced are chiefly hyperplastic, as few, if any, degenerative changes are apparent.

EXPERIMENTS WITH HÆMOLYTIC SERUM.

Hæmolytic serum was prepared by injecting a goose with normal defibrinated sheep's blood diluted with normal salt solution until it contained the same percentage of erythrocytes that is present in a corresponding injection of hæmolymph nodes.

The leucocyte curve (Table III) in this case is not very characteristic, consisting of a preliminary leucocytosis of about 2000 per cubic millimetre, followed by a still smaller increase and then a gradual rise covering a period of about seventy hours. By differential counts we find a typical "anticipatory" leucocytosis with its accompanying lymphopenia, after which there is a decrease in amphophiles and an increase in lymphocytes; the last two leucocytoses are due to the increase of lymphocytes.

The eosinophiles, after a preliminary decrease in number, gradually increase considerably above normal and then, as if a crisis had been reached, suddenly return to normal, at the end of about ninety-six hours.

The erythrocytes, after a rapid preliminary rise of one million, drop in twelve hours about two million and gradually decrease in the next thirty-two hours a million more, making a total destruction of about three million erythrocytes per cubic millimetre, or 28+% of the entire number of corpuscles.

SHEEP 9.—Received intraperitoneally 35 cc. of serum from Goose 7 at 11.30 A.M., February 9, 1904. At 3 P.M. the sheep appeared sick. The blood is dark in color; at 10.30 P.M. the blood is light in color and watery. February 11, at 5 P.M., one normoblast is found in a differential count of 500 cells. February 12, 9 A.M., two normoblasts are seen. February 14, 9 A.M., one normoblast is found. The sheep was killed February 15, 11.30 A.M. *Autopsy.*—There is

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no excess of peritoneal fluid. The hæmolymph glands are dark in color. The bone marrow is light in color. Axillary, mesenteric, and retroperitoneal lymph glands are dark in color. All other organs and tissues are apparently normal. There is no infection.

Histology.—The hæmolymph glands show few changes resulting from the injection of hæmolytic serum. The glands are somewhat congested and show evidences of increased erythrocyte destruction; large endothelial phagocytes are filled with pigment and partially broken-down corpuscles. Nuclear destruction in the lymphoid tissue is perhaps somewhat more abundant than normal, and large vesicular and in some cases pyknotic nuclei are present. The eosinophiles are increased in number.

The lymph glands show distinct evidences of proliferation in the germ centres, which are large and well developed. Evidences of erythrocyte destruction are much more marked than under normal conditions. Numerous large brown pigment granules are present, for the most part within phagocytes. Numerous vesicular and pyknotic nuclei and phagocytes containing broken nuclei are also present. There is some increase in eosinophiles.

The bone marrow shows an increase of mature amphophiles together with rather marked increase of the myelocytes. There is also a distinct increase of megaloblasts and normoblasts. In all of the tissues examined there are rather marked proliferative changes in the endothelium.

The injection of a hæmolytic serum into a sheep not only causes an increased destruction of erythrocytes, but has an effect on the leucocytes and indirectly at least on the leucocyte-forming organs. Although the changes in these organs are not pronounced, and in this case, at least, not very characteristic, they are nevertheless evident. The increase in both lymphatic and hæmolymphatic glands of endothelial phagocytes containing red cells and pigment is the most striking histological change found. In connection with these evidences of hæmolysis within cells is to be noted the increase of eosinophiles.

EXPERIMENTS WITH HÆMOLYMPHOCYTOTOXIC SERUM.

In two instances sheep were injected with hæmolymphocytotoxic serum. The first gave a more typical alteration of the leucocytes, but the animal was killed shortly after injection, in order to obtain tissues at an early stage; in the second animal the leucocytes were allowed to return to normal.

In these experiments changes undergone by the leucocytes (Tables IV and V) have resembled those which follow the injection of normal serum of the goose, but the increase which first

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occurs takes place in a shorter length of time, as if the demand has been more urgent and the tendency to proliferate given a sharper impetus. After the first rise there is a complete return to normal, followed by a second slower and slighter leucocytosis, which in turn, after twenty-four hours, is followed by a third rise.

On differential counts a typical "anticipatory" leucocytosis with its accompanying lymphopenia is observed. The first rise already described is due entirely to an amphophile leucocytosis; the second, however, is due to a lymphocytosis, and the third to a combined increase of both amphophiles and lymphocytes. It is readily seen that, with the exception of an "anticipatory" leucocytosis, these changes are almost identical with those produced by the injection of normal serum of the goose, until the third rise is reached. In the case of the normal serum this is due entirely to a lymphocytosis, while with the hæmolymphotoxic serum it is due to a combined rise of lymphocytes and amphophiles.

The large mononuclears remain practically unchanged throughout the experiment. It is noteworthy that in every case cited the eosinophiles, after a preliminary decrease, gradually reach a number considerably above normal and then, as by a crisis occurring at the end of about ninety-six hours, suddenly return to normal.

There is a marked difference between the erythrocyte curves in the two experiments. In the first there is a slight rise occurring within the first four hours and then a decided drop. Great emphasis is to be laid on this marked decrease in number of erythrocytes, their number dropping from 9,215,000 to 4,824,000 in four hours—a fall of 47 + % of entire number of corpuscles. In the second curve, however, there is a preliminary increase followed by a slight decrease, another rise, and then a decided drop from 15,760,000 to 13,400,000 in twelve hours, there being altogether a destruction of 2,360,000 corpuscles, or about 15% of the entire number present. In both instances after this marked decrease in number there is a gradual rise to normal, and in both instances this regeneration is somewhat excessive, the erythrocyte count exceeding normal.

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SHEEP 3.—Received 35 cc. serum from Goose 3 at 2 P.M., November 6, 1903. At the time of the count on November 6, at 10.30 P.M., the blood was noticeably lighter in color and more watery than normal. The animal was killed November 10, at 10 A.M. *Autopsy* showed slight œdema around the site of inoculation. A large amount of fluid was present in the peritoneal cavity. All of the lymph glands, with the exception of the hæmolymph glands, were large, hard, and extremely dark in color.

The spleen is small, being not over two-thirds its normal size. One kidney is markedly atrophic, the other showing compensatory hypertrophy. On section the pelvis is large and dilated; the cortex is thin. The liver is large, dark in color, and bleeds freely on section. The bone marrow is normal in appearance. There is no infection.

Histology.—The hæmolymph glands show a disappearance, or at least a diminution, in size and numbers of the distinct proliferating germ centres found in normal glands. In twenty-five glands examined exceedingly few germ centres are found, and those present are small and show few mitotic figures. The blood sinuses are somewhat congested and contain large numbers of leucocytes. Eosinophile cells (Fig. 2) are present in the lymphoid tissue in large numbers; most of them are polymorphonuclear, but some are mononuclear, and a few even polynuclear. There is less evidence of cell destruction in the hæmolymph glands than might be expected, only a few large vacuolated and some pyknotic nuclei being seen. Abundant endothelial proliferation is found, and large phagocytes containing broken-down erythrocytes and blood pigment are present in great numbers.

The germ centres in the lymph glands, while more numerous than in the hæmolymph glands, are small and do not show active proliferation. There is, however, an enormous amount of proliferation in the endothelium. The amount of blood pigment in some of the lymph glands is enormous, giving the glands on macroscopical examination a dark brown or blackish color. The pigment on microscopical examination (Fig. 3) is found in the medullary portion of the gland and is chiefly intracellular, having been taken up by phagocytes. It does not contain iron, and is probably hæmatoidin formed as the result of erythrocyte destruction. Other evidences of cell destruction are not more frequently observed than in the hæmolymph glands. There is considerable increase in the number of eosinophiles present.

In the spleen, as in the other tissues examined, the germ centres are small and are not actively proliferating. The spleen is very markedly atrophic, but this atrophy must have antedated the time of injection. Some pigment is present which, in sharp contrast to that found in the lymph glands, is iron-containing and chiefly extracellular.

The bone marrow shows a marked diminution in the number of mature cells, i. e., cells ready to enter the circulation. The marrow cells, with few exceptions, however, are in some stage of mitotic division. Megaloblasts and normoblasts are also increased in numbers, the former showing active proliferative changes. The endothelium is proliferating actively. Eosinophiles are decreased in numbers. A few large vacuolated nuclei are to be seen.

SHEEP 6.—Received 35 cc. serum from Goose 6 at 1 P.M., January 22, 1904. There was evidence of hæmoglobinuria at the end of forty-eight hours. The

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animal was killed January 29, 10 A.M.; at autopsy the organs were apparently normal.

Histology.—Since the sheep was killed seven days after injection, the tissues may be expected to show either a greater attempt at repair or a later stage of the intoxication.

The hæmolymph glands in this experiment, as in the last, show a decided diminution in size and numbers of germ centres. Evidences of cell destruction here, however, are very striking. With very few exceptions every nucleus, though not necrotic, is in some stage of fragmentation. Pyknotic, large vesicular, and vacuolated nuclei are in great abundance. Numerous phagocytes containing particles of broken-up nuclei are present. The pigment, phagocytes, and eosinophiles have the same characters as in Sheep 3.

The lymph glands show the changes described for Sheep 3, except that there is more evidence of nuclear destruction; there are vacuolated, vesicular, and pyknotic nuclei, and endothelial phagocytes are found. Blood pigment is present in considerable amount, but not to the same extent as in Sheep 3, although the phagocytes are as numerous.

The spleen in this case shows more marked nuclear destruction than in Sheep 3. A much larger amount of pigment (Fig. 4) is, however, present here; it is iron-containing, and is probably hæmosiderin.

The red bone marrow is not abundant. Proliferative changes are confined chiefly to the megaloblasts. The myelocytes are not numerous, and those present do not show very active mitosis. Fully developed amphophiles are, however, numerous. Eosinophile cells are decreased in number.

After the injection of hæmolymphotoxic serum there is, as with the injection of normal serum from the goose, a sharp demand on the leucocyte-forming organs, which is responded to first by the bone marrow and later by the lymphocyte-forming organs. The bone marrow is able, in some degree, to compensate for this loss by proliferation, but the lymphocytes in the blood seem to be solely supplied by preformed cells. Histological examination seems to show that the tendency to proliferation of the cells antecedent to the lymphocytes is inhibited. This inhibitory or toxic action, while slight at first, becomes exceedingly destructive, as shown by the marked signs of degeneration in the lymphadenoid tissue.

The differences in changes affecting the erythrocytes in the two experiments may be partly due to the pathological condition of the kidneys in Sheep 3. In both experiments there has been a preliminary rise in the number of erythrocytes, then a fall, and finally a slow recovery which becomes excessive and carries the erythrocyte curve above normal. In Sheep 3, the

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kidneys being pathological, it is probable that the excretion of the toxin has not been carried on to the same extent as in Sheep 6. Since this animal has been able to excrete some part of the toxin, the effect has occurred slowly and is less marked.

Evidence of erythrocyte destruction in the tissues is somewhat more marked in Sheep 3 than in Sheep 6, but this difference again is partly referable to the kidneys of the former; for in the latter hæmoglobinuria was present, while in Sheep 3 there was no evidence of it. The regeneration of erythrocytes is explained by the marked proliferation of megaloblasts in the bone marrow.

PARALLEL EXPERIMENTS WITH HÆMOLYMPHOTOXIN AND WITH HÆMOLYSIN.

Control experiments were here tried with hæmolymphotoxic and erythrolytic serums with the special purpose of determining the amount of hæmolysis in either case and of making careful comparisons.

To this end for corresponding experiments geese and sheep were selected respectively as nearly alike as possible; they had been kept under similar conditions. The geese were injected with the same amount of fluid in each case, and each injection contained the same amount of blood as determined by Gower's hæmoglobinometer; the goose injected with emulsion of hæmolymph gland received the same number of erythrocytes as the one receiving the blood. The conditions of the experiments were made parallel in every respect, so that the results obtained might be exactly compared.

I. *Experiment with Hæmolymphotoxin.*—Changes in the number of leucocytes (Table VI) obtained by injecting hæmolymphotoxin from the goose selected resemble in general those of Table IV, obtained after injection of hæmolymphotoxin from Goose 3; twice their number rises markedly. Examination of differential counts shows that the preliminary leucocytosis in both cases is due at first to an amphophile leucocytosis, later aided by a lymphocytosis. The second rise, however, is markedly different in the two cases, for in one instance it is

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entirely due to an amphophile leucocytosis, while in the other it is wholly a lymphocytosis.

The erythrocytes in Sheep 12 after a preliminary rapid rise gradually but steadily decrease for fifty-six hours and then as gradually return to normal. Altogether there is a drop from 4,800,000 to 3,240,000, or a total destruction of 32% of the entire number of erythrocytes present.

The eosinophiles show the usual preliminary decrease, the gradual rise, and the so-called crisis.

SHEEP 12.—Received 40 cc. serum from Goose 12 at 1 P.M., April 23, 1904. Killed April 28, 4 P.M. *Autopsy.*—Hæmolymph glands are not numerous, but are large; lymph glands are rather dark in color. The liver has a peculiar greenish tinge. The bone marrow is anæmic. Otherwise the organs are normal and there is no infection.

Histology.—In sharp contrast to the other instance in which hæmolymphocytotoxin has been injected, this one shows the germ centres in the hæmolymph glands to be large, numerous, and actively proliferating. In other respects, however, they resemble those already described, in that there are a large number of degenerative changes, marked proliferation of endothelium, many phagocytes containing blood pigment and corpuscles, and a marked increase of eosinophiles.

In the lymph glands again the germ centres are large, numerous, and actively proliferating; otherwise they resemble those heretofore described. The amount of blood pigment and the number of phagocytes present in some of the glands is very large.

The spleen resembles those previously described very closely, except that there is a very little blood pigment present. Part of this pigment is hæmotoidin and is intracellular, while a part is hæmosiderin, and, situated chiefly outside the cells, is present in large amount.

The bone marrow is the seat of active proliferation and contains large numbers of mature cells, i. e., fully developed amphophiles.

II. *Experiment with Hæmolysin.*—In the sheep which received hæmolytic serum from Goose 14, as in the other experiment with hæmolytic serum, the leucocytes do not show very characteristic changes (Table VII); their number undergoes two slight but rather prolonged rises and falls. These changes are almost wholly referable to the amphophiles, the lymphocytes varying only slightly throughout the experiment.

The erythrocytes decrease slightly at first and then gradually rise to a number somewhat above normal. This number then suddenly drops from 5,340,000 to 4,280,000 in twelve hours,

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1,060,000 erythrocytes, or 19% of the entire number in circulation, being destroyed.

SHEEP 14.—Received 40 cc. serum from Goose 14, on April 23, 1904, at 2 P.M. No erythroblasts were found in any of the preparations of blood. The animal was killed April 28, 3 P.M. At autopsy a large amount of peritoneal fluid is found. Hæmolymph glands are not numerous but are large. The spleen is slightly smaller than normal. The lungs are anæmic. Otherwise the organs are normal; there is no infection.

Histology.—The histological changes resemble very closely those already found after the injection of hæmolytic serum.

The two preceding experiments vary but little from other similar experiments already described, except that heretofore the effect of the hæmolymphotoxin has been to inhibit proliferation in the germ centres; in Sheep 12 proliferation is very active.

Under the same conditions, the serum obtained by the injection of emulsion of hæmolymph glands is more hæmolytic than that obtained by the injection of a corresponding amount of erythrocytes; in the first case there is a destruction of 32% of the entire number of erythrocytes in circulation, while in the latter there is a destruction of only 19%.

EXPERIMENTS WITH HEATED HÆMOLYMPHOCYTOTOXIN.

In the following experiment hæmolymphocytotoxin was heated to 56° C. for half an hour to destroy the hæmolytic action before injection.

Changes in blood (Table VIII) obtained in this experiment are strikingly like those obtained by Bunting after the injection of lymphotoxin. There is first a leucocytosis of moderate extent due to a combined increase of amphophiles and lymphocytes. Following this there is a still greater leucocytosis and finally a more moderate one. All three leucocytoses are due to a combined rise of both amphophiles and lymphocytes. They differ from those of other experiments by the sharp rise and fall of the lymphocytes which ordinarily rise slowly to a certain height and then gradually decline.

The erythrocytes show, at first, a slight decrease, due to the

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fact that the hæmolytic action of the serum was not completely destroyed, but they soon regain their normal height, which they retain throughout the experiment. The large mononuclears vary little; the eosinophile cells show the ordinary preliminary increase and crisis as described above.

SHEEP 4.—Received 30 cc. of serum from Goose 4. at 3 P.M., November 18, 1903. This serum had, previous to injection, been heated to 56° C. for half an hour. The animal was killed 10 A.M., November 22. At autopsy slight œdema is found around the place of inoculation. There is a large amount of peritoneal fluid. The hæmolymph glands are dark in color and apparently not so large nor so numerous as normal. The spleen on section is dark in color, but is otherwise normal. All other organs and tissue are apparently normal; there is no infection.

Histology.—The hæmolymph glands are congested, the sinuses being large and densely crowded with erythrocytes. Germ centres are few in numbers, and when found are small but show fairly active proliferation. Evidences of cell destruction are not marked, although a few pyknotic and vesicular nuclei are to be found. Some blood pigment, probably hæmatoidin, is present. Eosinophile cells are present in great numbers and are both of mononuclear and polynuclear type, but are fewer than in Sheep 3.

Some of the lymph glands, particularly the mesenteric glands, show toxic changes, manifested by more or less wedge-shaped areas of necrosis (Fig. 1) extending into the lymph glands with their bases toward the periphery. These areas appear as granular eosin-staining masses around which in some cases there is a beginning attempt at sequestration by leucocytes. Phagocytes containing fragmented nuclei are very abundant. The germ centres, while not large, show fairly active proliferative changes. Some blood pigment, probably hæmatoidin, is present, and is contained entirely in large phagocytes. There is an enormous increase in eosinophiles, both mononuclear and polynuclear.

The spleen is congested and shows signs of degeneration, pyknotic and fragmented nuclei being present. There is a large increase of eosinophiles. A small amount of blood pigment is present.

The changes in the bone marrow consist chiefly in a marked increase in number of the myelocytes. Mature amphophiles are also numerous, eosinophiles are decreased in numbers, and lymphocytes are numerous. Throughout all the tissues examined there is a marked proliferation of endothelium.

The kidneys in this animal, in contrast to the others cited, show the presence of iron situated in the tubules and demonstrable by the appropriate reaction.

Histological examination shows that the amphophile leucocytosis is due to the proliferation of cells in the bone marrow antecedent to the amphophiles, while the increase of lymphocytes is due almost entirely to the existence of preformed cells, since proliferation in the germ centres is not marked.

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A second experiment was conducted in exactly the same way as the preceding; the serum of a goose which had been immunized to hæmolymph glands being heated to 56° C. for a period of half an hour. It was expected that at the expiration of this time the hæmolytic property, at least that due to immunization with injected erythrocytes, would be destroyed.

Changes in the blood (Table IX) following the injection of this serum into the sheep resemble in only one point those obtained in the preceding experiment; there is at first leucopenia followed by a slight, gradual leucocytosis, after which there is a slight drop, another slight rise, and then a gradual return to normal. Changes affecting the entire number of leucocytes are closely followed by those of the lymphocytes, the amphophiles being so few in numbers that they have little effect upon these changes.

The erythrocytes undergo a slight preliminary rise of 700,000 and then a sharp fall of two million, after which they gradually return to normal. The eosinophiles slowly but markedly increase until the sixty-fourth hour, when the so-called crisis occurs. The large mononucleus and mast cells show no noteworthy alteration.

SHEEP 19.—Received 45 cc. of serum from Goose 19 on May 16, 1904, at 1 P.M. The serum previous to injection had been heated to 56° C. for half an hour. Sheep 19 was killed at 10 A.M., May 21. There was a moderate amount of peritoneal fluid. The hæmolymph glands were rather large and numerous. All the organs were apparently normal. Bone marrow was light in color. There was no infection.

Histology.—The hæmolymph glands are congested, the sinuses being large and densely crowded with erythrocytes. The germ centres are large and actively proliferating. There is, moreover, a slight general hyperplasia of lymphoid cells. There is little evidence of degeneration. Endothelial proliferation is marked. Large numbers of phagocytes containing entire corpuscles and some containing blood pigment, hæmatoidin, are to be seen. There is an enormous increase of eosinophiles.

The germ centres in the lymph glands are also large and actively proliferating.

In this case no necrotic areas, similar to those observed in the previous experiment, are found. Degenerative changes are fairly numerous, vacuolar, vesicular, and some pyknotic nuclei being observed.

Large numbers of phagocytes containing blood pigment, hæmatoidin, are to be seen. Eosinophiles are increased in numbers. The spleen is normal,

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except for the presence of a large amount of blood pigment which, situated outside of the cells, is for the most part hæmosiderin. Mitosis in the germ centres is active, and there is a rather marked proliferation of the endothelium. Eosinophiles are somewhat increased.

In contrast to the previous experiment, the kidney tubules do not show the presence of iron.

The bone marrow seems to be little affected, and is practically normal. There is, however, some increase of erythrocytes and of eosinophiles. The presence of large numbers of mature amphophiles is noteworthy when it is recalled that few were present in the circulation.

This experiment differs markedly from the one preceding it, the changes not only in the blood but in the tissues being different.

These experiments suggest, in accordance with the statement of Pearce, that necrosis is not a specific action of the serum, but is due to its erythrolytic and agglutinative properties.

The marked hæmolysis which occurred in the last experiment is, however, of great importance, for it tends to show the presence of some hæmolytic factor, which is resistant to heat and not due to immunization with erythrocytes alone.

EXPERIMENTS WITH HETEROHÆMOLYMPHOTOXIN.

In the following experiment a hæmolymphotoxin for the sheep was prepared by the usual method, and was then injected into a rabbit in order to determine if a hæmolymphotoxin active for one animal would have the same action in an animal of a different species or if its action would be peculiar to the species for which it was prepared. Following this injection (Table X) there is an extremely marked preliminary leucopenia showing that the peritoneal irritation has been extreme. This leucopenia is followed by a well-developed leucocytosis, which is in turn followed by a decided drop in the number of leucocytes, interrupted by one slight rise; the number of leucocytes then gradually returns to normal. The amphophiles rise steadily for twenty hours, after which they as steadily decrease, rising to normal just before death. The preliminary decrease of lymphocytes is well-marked and of long duration; these cells do not begin to increase in number until the twentieth hour, after which they very closely follow the total number of leucocytes.

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It would appear, then, as if proliferation in the lymphocyte-forming organs had received a check, or that amœboid movement of the fully developed cells in the lymph glands and spleen was slower or more difficult than usual.

The eosinophiles follow the usual course observed after the injection of hæmolymphotoxin, decreasing at first and then rising until the forty-fourth hour, when the so-called crisis occurs.

Changes undergone by the erythrocytes are of great interest since they give evidence of very marked hæmolytic action. There is at first a sharp rise of about one million, and then in the next five hours a decided drop of over three millions. Subsequently the number fluctuates as if the destruction at times overcame the supply, but steadily returns to normal.

RABBIT 1.—Received 10 cc. of serum from Goose 12 at 12 noon, April 23, 1904 Killed, 1 P.M., April 28. At autopsy the organs were found to be normal; there was no infection. No hæmolymph glands were found at autopsy.

Histology.—The germ centres in the lymph glands are fairly well developed, and proliferation is active. Mitotic figures are fairly numerous throughout the gland. Degenerative changes also are very abundant, many large vacuolar, vesicular, pyknotic, and fragmenting nuclei being present. Endothelial proliferation is also very marked, and many large endothelial phagocytes contain particles of pigment, probably hæmotoidin, while some contain fragments of nuclei. Eosinophiles are greatly increased in numbers.

Proliferative changes in the spleen are less evident than in the lymph glands, but there is very marked endothelial proliferation. The blood pigment here present in rather large amounts is hæmatoidin. Eosinophiles are markedly increased in numbers.

The cellular elements throughout the bone marrow are undergoing proliferation; the endothelium is also the seat of proliferation.

Comparison of the histological changes just described and those produced by the injection of hæmolymphotoxin into a sheep shows that they are almost identical.

Difference in the changes affecting the blood and perhaps the slightly more toxic effect which the serum seems to have on the rabbit may be explained by the fact that a greater amount of serum per kilo of body weight was injected into the rabbit. Moreover, the hæmolytic effect on the rabbit is very marked, a destruction of 43% of the total number of erythrocytes in circulation having occurred in five hours.

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The eosinophiles of the rabbit, like those of the animal for which the hæmolymphotoxin was prepared, are increased greatly in numbers. It is probable, then, that the toxin produced by the injection of emulsions of hæmolymph glands into an intermediary animal is heterotoxic, the changes in the two species employed being the same in every respect.

SUMMARY OF EXPERIMENTS WITH HÆMOLYMPHOCYTOTOXIC SERUM.

After the injection of the serum of an animal immunized against hæmolymph glands there is a series of changes which may be classified in several groups.

I. *Changes Due to Hæmolytic Properties.*—The hæmolytic properties of the so-called hæmolymphotoxin are very marked and their effects are observable not only in the blood counts, but also in the tissues. Its hæmolytic power is in fact stronger than that of the serum obtained when the same number of corpuscles are injected into the intermediary animal as are contained in the emulsion of hæmolymph glands employed. This fact is demonstrated by the following table in which parallel experiments are compared:

(*a*) Normal serum caused an increase of 1,000,000 erythrocytes.

(*b*) 1-Hæmolytic serum caused the destruction of 3,000,000 erythrocytes, or 28% of the entire number in circulation.

2-Hæmolymphotoxic serum caused the destruction of 4,392,000 erythrocytes, or 47% of erythrocytes in circulation.

(*c*) 1-Hæmolytic serum caused the destruction of 1,060,000, or 19% of erythrocytes in circulation.

2-Hæmolymphotoxic serum destroyed 1,560,000, or 32% of erythrocytes in circulation.

There is, therefore, a difference in one case of 19% and in the other of 13% between parallel experiments. Taking into consideration the fact that in one case the goose serum may have had a stronger inherent tendency to destroy erythrocytes than in the other and the possible percentage of error, it is still impossible to explain the difference between the two experiments.

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Moreover, when the serum was heated to a temperature sufficient to destroy all hæmolytic action in a serum produced by the injection of erythrocytes alone, there was still considerable destruction of erythrocytes after injection into the sheep. Furthermore, on injection of the toxin into an animal of a different species the hæmolytic effect was very marked—more marked than would usually follow the injection of hæmolytic serum produced with erythrocytes alone.

Taking all these facts into consideration, it seems probable that the hæmolymphotoxin has some hæmolytic action not due to the reaction of the goose to erythrocytes alone.

II. *Changes Due to Lymphotoxic Properties.*—Certain of the changes produced by the injection of hæmolymphotoxin are referable to the immunization of the goose against the lymphoid tissue in the hæmolymph glands.

In one experiment there is apparently an inhibition of proliferation in the germ centres, while in the other two proliferation is marked. This occurrence of proliferation agrees with results generally obtained after injection of lymphotoxin. While proliferation is not necessarily due to the direct action of the toxin, in most instances at least no inhibition of proliferation is caused by hæmolymphotoxin.

The changes affecting the leucocytes of the blood and to a certain degree the proliferative changes in the blood-forming organs are due to the peritoneal irritation following the injection of the toxin; to a certain extent they are referable to the direct effect of the toxin.

The degenerative changes which occur are, however, due to the lymphotoxic action, and those following the injection of hæmolymphotoxin are in all respects like those following the use of lymphotoxin.

III. *Changes Due to Endotheliotoxic or Phagocytoxic Properties.*—In order to demonstrate the presence of an endotheliotoxin or phagocytotoxin a comparison may be made of the number of phagocytes present in the spleen and lymph glands after the injection of hæmolymphotoxin, lymphotoxin, and hæmolysin.

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Such a toxin is doubtless present in the hæmolymphotoxic serum employed for it is readily seen, even when a low-power objective is used, that the number of phagocytes present after the injection of hæmolymphotoxin is much greater than after the injection of hæmolysin. That their presence, moreover, is not secondary to the destruction of erythrocytes is shown by the fact that the number of phagocytes found after the injection of hæmolymphotoxin is greater absolutely than the number present after the injection of hæmolysin, while with hæmolymphotoxin the phagocytes are increased out of proportion to the number of red blood cells destroyed. There is further proof that this accumulation of phagocytes is not secondary to destruction of erythrocytes; Warthin (9) has described similar changes in hæmolymph glands after removal of the spleen.

It is probable, therefore, that an endotheliotoxin or phagocytotoxin is present in the serum.

IV. *Changes Due to Hæmolymphotoxic Properties.*—Is it necessary to assume the existence of a specific hæmolymphotoxin in order to explain the results obtained?

In the first place, there is produced a certain amount of hæmolysis which is not due alone to the erythrocytes injected into the goose.

One theory regarding the action of hæmolymph glands is that they are hæmolytic. Such hæmolysis may be explained in two ways: (1) it is due to a ferment, secreted by phagocytes; (2) the phagocytes take up and remove corpuscles bodily from the circulation.

If the first hypothesis is correct, it is evident that in immunizing the goose with emulsions of hæmolymph glands this destructive action upon erythrocytes must be overcome as well as the native resistance of the erythrocytes themselves; so that, in the end, the goose serum would become more destructive to erythrocytes than if the erythrocytes contained in the emulsions of hæmolymph glands had been alone injected.

The changes which occur in the sheep injected with hæmolymphotoxic serum are referable to substances produced during the reaction of the goose to the tissues injected into it, namely,

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lymphoid tissue, endothelium, etc., and to the peritoneal irritation in the sheep due to the injection of the serum; hence it is unnecessary to assume the existence of a specific hæmolymphotoxin.

V. *Heterotoxic Properties.*—The serum obtained after the injection of emulsions of hæmolymph glands into an intermediary animal is heterotoxic, for when it is injected into an animal of a different species every detail of the process is duplicated and consequently it is not specific for the primary species alone.

CONCLUSIONS REGARDING HÆMOLYMPHOTOXIN.

(1) The toxin produced by the injection of emulsions of hæmolymph nodes from one species into an intermediary animal is complex in composition, having lymphotoxic, endotheliotoxic or phagocytotoxic and hæmolytic properties.

(2) These toxins when so obtained are heterotoxic, affecting other species besides that one which was used for the immunization of the intermediary animal.

(3) There is no specific hæmolymphotoxin.

(4) The chief function of the hæmolymph glands is hæmolysis; they remove partially broken-down erythrocytes from the circulation.

(5) Hæmolysis is probably brought about by a ferment produced in the hæmolymph node.

(6) This ferment is aided by phagocytes which remove the debris from the glands.

(7) The ferment is probably produced by the endothelium.

(8) In this connection it is desirable to emphasize the theory that hæmolymph glands have a part in the production of eosinophiles, the granules arising from products of disintegration of red cells. Such material is taken up by mononuclear leucocytes the nuclei of which become polymorphous. In almost every experiment there has been some destruction of erythrocytes and there has been an increase of eosinophiles in the blood and lymphoid structures in proportion to this destruction. Furthermore, when, in making special stains for eosinophiles, the cells were overstained with eosin and part of the eosin was afterward

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removed, the phagocytes containing particles of erythrocytes tended to hold the stain longer than either ordinary phagocytes or normal erythrocytes.

After the preceding conclusions concerning hæmolymphotoxin have been reached, it becomes necessary to study carefully by the same methods other so-called cytotoxins in order to ascertain to what extent the changes produced by them vary from those already described and what degree of specificity each possesses.

EXPERIMENTS WITH ENDOTHELIOTOXIN.

So far as can be ascertained, no attempt has heretofore been made to produce a pure endotheliotoxin. In order to produce such a toxin the aortas of sheep were removed and the endothelium, under as nearly sterile conditions as possible, was scraped off and made into an emulsion in sterile physiological salt solution. Great care was necessary to avoid the removal of connective tissue and the entrance of blood into the emulsion.

The toxin produced by the injection of this emulsion into a goose gave, on being injected into a sheep, a rather typical blood picture (Table XI). There is at first a rather sharp leucopenia, followed by a leucocytosis, reaching its summit at the end of about twenty hours. The number of leucocytes then gradually decreases and again rises, producing a leucocytosis which, of greater height than the first, keeps its elevation for about twenty-five hours, the number then returning to normal. After the so-called "anticipatory" leucocytosis the changes in the total number of leucocytes are very closely followed by those of the lymphocytes, the amphophiles after the first rise markedly decreasing and then very gradually rising to a level somewhat above normal, to decrease again sharply.

Changes in the number of erythrocytes are noteworthy; from the first, with the exception of a slight rise at the end of the sixty-sixth hour, it gradually yet steadily decreases, falling from 5,160,000 before injection to 2,760,000 at time of death—a destruction of 2,400,000 corpuscles or over 46% of entire number of erythrocytes in circulation.

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SHEEP 15.—Received 40 cc. of serum from Goose 15 at 1.30 P.M., April 9, 19. One nucleated red blood corpuscle was seen in counting five hundred cells on April 10, at 9.30 P.M. The animal was killed April 15, 10.30 A.M. At autopsy the entire body is anæmic, though fairly well nourished. A large amount of intraperitoneal fluid is present. The hæmolymph glands are numerous and hyperplastic. The liver and kidneys are normal. Spleen is slightly smaller than normal. The heart and lungs are normal. The bone marrow is fairly red. There is no infection.

Histology.—The germ centres in the hæmolymph glands are more numerous and larger than in any experiment heretofore examined. Proliferation in the germ centres is exceedingly active. Many large phagocytes filled with blood pigment or with entire red blood corpuscles are to be seen. Endothelial proliferation is very marked. Evidence of degeneration is rarely found. Eosinophiles are somewhat increased in numbers.

In the lymph glands, as well as in the hæmolymph glands, the germ centres are large, numerous, and show signs of very active proliferation. Proliferation of endothelium is also very marked, and large numbers of endothelial phagocytes containing blood pigment, probably hæmatoidin, are to be seen. On the other hand, degenerative changes are here very numerous, many vesicular, some vacuolar, and a few pyknotic nuclei being found. Eosinophiles are normal.

The spleen is slightly smaller than normal. The germ centres in the spleen are large and are the seat of active proliferation. Endothelial cells have undergone proliferation and many endothelial phagocytes containing blood pigment, probably in this organ also hæmatoidin, are to be seen. Degenerative changes are marked.

Proliferative changes in the bone marrow are widespread, every type of cell being involved in the process, which affects particularly the endothelium, so that large endothelial cells may be seen in every stage of multiplication. The myelocytes and erythroblasts are also greatly increased in number. Large numbers of mature cells such as amphophiles are present.

The liver shows a slight fatty degeneration and the kidney a slight amount of cloudy swelling.

The results obtained in the preceding experiment are important because they demonstrate the occurrence of marked changes not only in the blood but in the blood-forming organs. The marked and steady decrease in the number of erythrocytes after the injection of the toxin indicates that it is possible for the endothelium alone, through its phagocytic tendencies, to cause a profound and perhaps lasting anæmia. It cannot, however, be maintained that this destruction of erythrocytes is due to phagocytic action alone, for the histological changes will not justify such a statement. It is not improbable that a large part of it is due to the secretion of some ferment, such as that present in

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the hæmolymph glands; it destroys the erythrocytes and causes their removal from the circulation.

EXPERIMENTS WITH HEPATOTOXIN.

In 1900, Delezenne (11) obtained a *serum antihepatique* by injecting emulsions of the liver of dogs into rabbits and ducks. A strongly toxic serum causing death in doses of 4 cc. per kilo of body weight was obtained. At autopsy, the liver was yellow, friable, and shrivelled, the animal having apparently died with a condition resembling that produced by phosphorus. By microscopical examination the cells were found to be markedly degenerated. In certain areas the lesion was more advanced and the protoplasm of the cells had in great part disappeared. The blood-vessels were dilated and capillaries filled with blood separated the rows of degenerated hepatic cells. There was an infiltration of leucocytes throughout the lobules. When the process was less acute, the animal lived from five to fifteen days, and marked fatty degeneration of the liver alone was noted.

Pearce (12) produced hepatotoxins with emulsions made from liver tissue washed entirely free of blood and with unwashed liver. After injection of hepatotoxins prepared with unwashed liver he found no changes in the hepatic cells which could be ascribed to their specific action. With sera from washed livers he found increased granulation of the liver cells, with slight, fatty transformation. Necrotic areas were found in only one experiment in which a large dose of strongly hæmagglutinative serum was employed; he believes that these necrotic areas are due to thrombi formed by agglutinated red blood cells.

In the experiment to be described (Table XII) there are no characteristic changes in the blood. There is a sharp preliminary leucocytosis, followed by a step-like decrease in the number of leucocytes; subsequently there are two minor rises. The first leucocytosis is altogether due to an increase in amphophiles; the subsequent leucocytoses are due to an increase of both amphophiles and lymphocytes.

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The eosinophiles undergo a preliminary decrease, followed by a gradual rise and fall.

The erythrocytes exhibit rapid preliminary increase, followed by a rather sharp and well-marked fall, after which there is a return to normal, again followed by a very gradual yet distinct decrease.

SHEEP 16.—Received 35 cc. of hepatotoxic serum from Goose 16 at 1 P.M., April 5, 1904. Killed, 11 A.M., April 11. At autopsy the hæmolymph glands are found to be few in number. The spleen is slightly smaller than normal. There is an elevated white nodule, 5 x 5 x 5 mm. on the liver. The liver is light in color and apparently contains fat. The spleen is dark in color. The other organs are normal. There is no infection.

Histology.—The germ centres in the hæmolymph glands are large, and are the seat of active though not excessive proliferation. There is little evidence of degeneration, although some pyknotic and vesicular nuclei are to be seen. There is some proliferation of endothelium. Eosinophiles are decreased in number.

The lymph glands show no changes beyond a slight increase of proliferation in the germ centres and endothelium, and the presence of a considerable amount of blood pigment, probably hæmatoidin, contained for the most part within endothelial phagocytes.

The spleen is slightly smaller than normal and somewhat congested. There is increased proliferation both in the germ centres and in the endothelium. Some blood pigment is present, but it is entirely hæmatoidin, for no reaction for iron could be obtained.

The bone marrow is almost wholly normal, only a slight increase of myelocytes and erythroblasts being apparent.

The elevated white area on the liver noted at autopsy proved to be an encysted animal parasite. The liver is the seat of fairly advanced fatty degeneration. The kidney shows only a very slight cloudy swelling.

Changes in the blood, as before stated, are limited to the rather marked hæmolysis. Although an attempt was made to wash the blood from the liver before injecting it into the goose it was impossible to remove the blood entirely, so that hæmolysis is probably for the most part due to this factor. The histological changes are not characteristic; changes in the blood-forming organs are insignificant and the fatty change in the liver, while confirming the results obtained by Delezenne, is not so marked as that produced by some other cytotoxins. Nearly every cytotoxin causes at least a slight fatty degeneration of the liver. We may say, then, that the hepatotoxins have no specific action.

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EXPERIMENTS WITH SPLENOTOXIN.

Flexner (3) found after the injection of splenotoxin that the chief changes were in the lymph glands and spleen. In the lymph glands the germ centres were large and the cells had undergone degeneration, but few mitotic figures were present. In the spleen there was wide distension of the blood sinuses, slight general hyperplasia of splenic cells, and marked evidences of degeneration.

Changes in the blood (Table XIII) obtained in the following experiment are peculiar. There is at first a rather rapid rise and fall of the number of leucocytes, soon followed by a slighter rise and fall; there is then a period of about forty-eight hours during which the leucocytes vary but little; a marked increase in number occurs and is followed by a gradual decrease. The first increase is entirely due to an amphophile leucocytosis, there being at the same time the usual lymphopenia. The second increase is referable to the lymphocytes, while the third is again due to amphophiles.

The eosinophiles decrease at first and then gradually rise until the so-called crisis occurs at the end of thirty-six hours.

Changes affecting the erythrocytes are characteristic; hæmolysis causes the destruction of only 15% of the erythrocytes present in the circulating blood.

SHEEP 18.—Received 30 cc. of splenotoxic serum from Goose 18 at 1 P.M., April 14, 1904. Killed, April 21, 1.30 P.M.

Autopsy.—Hæmolymph glands are small but numerous. The spleen is smaller than normal. The kidneys are normal. The liver is light in color. The bone marrow is anæmic. Moderate amount of peritoneal fluid is present. The heart and lungs are normal. There is no infection.

Histology.—The hæmolymph glands are somewhat congested. Germ centres are few in number, and there is little proliferation of cells except in the endothelium. Degenerative changes are very numerous, and many vesicular and pyknotic nuclei are to be seen. Eosinophiles are decreased in numbers.

The germ centres in the lymph glands are sparsely scattered, but those which are found are large; they show many degenerative changes, the nuclei of the cells being swollen and vacuolar.

The spleen is small. There is a slight tendency to general hyperplasia of the lymphadenoid tissue, but more conspicuous is the marked degeneration, characterized by the presence of pyknotic and fragmented nuclei. In the

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hæmolymph glands, lymph glands, and spleen numerous endothelial phagocytes are found containing blood pigment, probably hæmatoidin, and nuclear debris.

The bone marrow contains much fat, and few changes from the normal are to be observed, except perhaps proliferation of endothelium.

The liver shows advanced fatty degeneration, the liver cells being occupied by large globules of fat. The distribution of fat is fairly uniform throughout the lobules.

The kidneys show slight cloudy swelling.

The changes observed in this experiment resemble very closely those described by Flexner. The toxin apparently has a specific effect upon the spleen, causing extensive degenerative changes; these changes, however, do not differ so much from those produced by other toxins that we are justified in assuming that the difference is one of kind and not of degree. The fatty degeneration of the liver is particularly noteworthy since it is greater in extent than that found after the injection of hepatotoxin, and therefore makes still more doubtful the specificity of that toxin.

EXPERIMENTS WITH NEPHROTOXIN.

In a recent article by Pearce (12) the subject of nephrotoxins is so carefully reviewed that it will be necessary here merely to describe their action upon the blood and blood-forming organs.

Changes in the number of leucocytes (Table XIV) are characterized by a preliminary leucopenia which is of long duration. This leucopenia is followed by a well-marked rise and fall and later by two lesser rises. The behavior of the leucocytes seems to depend upon the lymphocytes for, saving a slight rise in the number of amphophiles during the first leucocytosis, the changes of the total number of leucocytes and of lymphocytes are very similar.

The erythrocytes show at first a slight increase in number, and then a gradual decrease, showing that the serum is somewhat hæmolytic in spite of the fact that a large part of the red blood corpuscles have been washed out of the emulsion before it has been injected into the goose.

SHEEP 17.—Received 40 cc. of nephrotoxic serum from Goose 17 at 2 P.M., April 1, 1904. Killed, April 7, 11 A.M.

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Autopsy.—The hæmolymph glands are dark in color. The liver bleeds freely on section. There is well-marked congestion of the medullary portion of the kidney. All other organs are normal; there is no infection.

Histology.—The hæmolymph glands are fairly normal, having well-developed germ centres, which are the seat of active proliferation, and are in all respects normal. Endothelial phagocytes are somewhat more abundant than normally. They contain particles of blood pigment, probably hæmatoidin, and entire erythrocytes. Eosinophiles are normal in number. There is an increased proliferation of endothelium.

The germ centres in the lymph glands are large and actively proliferating. In the mesenteric glands in particular there is a considerable amount of blood pigment, probably hæmatoidin, contained in large phagocytes. There is considerable proliferation of endothelium shown by the presence of large, free, mononuclear endothelial cells, some of which are the phagocytes above described. The eosinophiles are slightly increased in numbers.

The spleen is little affected by the injection of nephrotoxin, and is normal save for a slightly increased proliferation of the endothelium. There is present a small quantity of free blood pigment, hæmosiderin.

The bone marrow is little altered. All the cellular elements, particularly the eosinophile cells, are undergoing proliferation, which is slightly more active than usual.

The congestion of the medullary part of the kidney, noted macroscopically, is in places so marked that the lumina of the tubules are encroached upon. There are a few hyaline casts. The cells of the tubules furthermore show some cloudy swelling and desquamation, which is most marked in the cortex, where some of the tubules are entirely filled with debris.

The changes produced by nephrotoxin in the blood and blood-forming organs are insignificant; those changes which occur, namely, proliferation in the germ centres and proliferation of the endothelium, are secondary, the one to the peritoneal irritation, and the other to destruction of erythrocytes.

The changes in the kidneys, however, are well marked and characterize the action of the serum.

EXPERIMENTS WITH LYMPHOTOXIN.

Peritoneal irritation following the injection of lymphotoxin prepared by the method already described is doubtless severe since there is a marked preliminary leucopenia (Table XV). This leucopenia is soon followed by a rather pronounced increase in the number of leucocytes, the bone marrow apparently responding to the demand upon it. The available supply having been exhausted, the leucocytes decrease and then rise again more

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slowly yet to a greater height; this increase of the total number is due to an increase both of amphophiles and lymphocytes. The slow response of the lymphocytes to a demand upon them is evident since the preliminary lymphopenia is marked and of considerable duration, the increase of lymphocytes being gradual until the final leucocytosis which is almost entirely due to lymphocytosis.

The erythrocytes increase at first and then gradually return to normal. The eosinophiles and large mononuclears show no noteworthy change.

SHEEP 11.—Received 30 cc. of lymphotoxic serum from Goose 11, March 25, 1904, at 2.30 P.M. The animal was killed March 31, at 10 A.M.

Autopsy.—There is a small amount of peritoneal fluid. The hæmolymph glands are small and few in numbers. The left kidney is slightly smaller than normal. The spleen is somewhat small. Lymph glands are numerous and rather hyperplastic. There is a considerable amount of pericardial fluid. Little fat is present throughout the body. The liver is normal. There is no infection.

Histology.—The hæmolymph glands are considerably congested and contain only a small amount of lymphoid tissue. The few germ centres which are present are, however, large and show active proliferative changes. There is also considerable proliferation of endothelium. The eosinophiles are few in number, being considerably less abundant than usual. There is some pyknosis.

The germ centres in the lymph glands are numerous and large, showing many mitotic figures. There is also evident proliferation of endothelium, and large numbers of endothelial phagocytes containing blood pigment, probably hæmatoidin, are present. Evidences of degeneration are limited to the presence of pyknotic, vesicular, and a few fragmented nuclei; there are no necrotic areas similar to those found by Bunting (4) after the injection of heated hæmolymphotoxic serum. The eosinophiles are decreased in numbers.

The spleen is somewhat atrophic. The germ centres are large, numerous, and the seat of active proliferation. A considerable amount of both extracellular and intracellular pigments is present, and is in great part, if not entirely, hæmosiderin. There is little evidence of degeneration. Eosinophiles are decreased in number.

The cellular elements of the bone marrow are markedly depleted. Those which are present are, however, undergoing marked proliferation. The myelocytes exhibit most active proliferation, the erythroblasts stand next in order, and the eosinophiles show the fewest mitotic figures. Fully-developed cells are few in numbers, but a few amphophiles are present.

The changes just described differ markedly from those obtained by Bunting, and resemble more closely those observed

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by Flexner. They do not have a specific character, nor do they vary greatly from the changes observed after the injection of other cytotoxins.

SUMMARY OF CYTOTOXIC EXPERIMENTS.

Are the cytotoxins specific — that is, do they individually produce characteristic changes or do the changes which they produce differ in degree alone?

(1) Changes in the blood observed in the preceding experiments are in no instance characteristic and may be explained by the action on the blood and blood-forming organs, of the peritoneal irritation produced by the injection of the toxin. These changes do not vary sufficiently from those obtained by the injection of salt solution and of normal serum to justify the assumption that they show, in any case, anything specific, particularly when we take into consideration the different conditions under which the animals may have been at time of injection.

(2) Histologically, certain differences are evident, but again all of these are of degree and not of kind. For instance, fatty degeneration of the liver was found after injection of hepatotoxin, but in almost every case examined there was slight fatty degeneration, while in the case of splenotoxin the fatty change was much greater than after hepatotoxin.

In every experiment almost the same changes were observed; they may be classified as follows:

Hæmolymph Glands.—Proliferation in the germ centres and endothelium; large numbers of endothelial phagocytes containing blood pigment, probably hæmatoidin; rather marked degenerative changes.

Spleen.—Proliferation in the germ centres and endothelium; some phagocytes containing blood pigment and usually more or less free iron-containing pigment, probably hæmosiderin.

Bone Marrow.—Proliferation of all elements.

Kidney.—Slight cloudy swelling.

Liver.—Slight fatty degeneration.

Throughout the entire series of injections these changes are

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to be observed and they vary merely in degree. All the cytotoxic sera are therefore hæmolytic, endotheliolytic, lymphotoxic, myelotoxic, etc.

When we take into consideration the severity of the changes which occur, the various sera may be arranged in the following order:

(*a*) Hæmolymphotoxin,
(*b*) Splenotoxin,
(*c*) Lymphotoxin,
(*d*) Endotheliotoxin,
(*e*) Nephrotoxin,
(*f*) Hepatotoxin.

It is readily seen that the above order takes into consideration the amount of hæmolysis produced by each serum. In the case of the spleen and the hæmolymph glands it is practically impossible to remove this factor, and in making the above classification the other factors have been given greater importance than this one. We have, then, in every case, a serum which causes a certain series of changes which always vary as to degree but not as to kind.

(3) The nephrotoxin perhaps comes nearest to being specific, since macroscopically and microscopically marked congestion of the medullary portion of the kidney and pronounced cloudy swelling in the cortex were observed. Similar changes of the same severity were found with no other serum, and it appears that the renal toxin had a specific, perhaps merely irritating, action on the kidney itself.

(4) It seems, furthermore, that the hæmolymph glands play some important part in the production of eosinophile cells, for in no case was there such a marked increase of these cells as after the injection of hæmolymphotoxin; indeed, after most of the toxins used, the number of eosinophile cells was found decreased.

CONCLUSIONS REGARDING CYTOTOXINS.

(1) The cytotoxins are not specific.

(2) The changes occurring after the injection of different cytotoxins are similar and do not vary in kind.

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(3) The changes do, however, vary in degree in the following order, the one having the most marked effect being placed first, etc.:

(*a*) Hæmolymphotoxin,
(*b*) Splenotoxin,
(*c*) Lymphotoxin,
(*d*) Endotheliotoxin,
(*e*) Nephrotoxin,
(*f*) Hepatotoxin.

(4) Nephrotoxin causes the most specific change.

(5) The hæmolymph glands play some important part in the production of eosinophiles. The hæmolytic action of hæmolymphotoxic serum is greater than that of other cytotoxic sera.

(6) The histological changes which are observed in every case are secondary to the hæmolytic factor present in all cytotoxic sera.

In conclusion I wish to express my thanks to Dr. Warthin, in whose laboratory and under whose directions these experiments were conducted, for his aid and encouragement in the work.

DESCRIPTION OF PLATES.

Plate IV.

Fig. 1.—Area of necrosis in lymph gland of Sheep 4 after injection of heated hæmolymphocytotoxin. Stained with hæmatoxylin and eosin.

Fig. 2.—Increase of eosinophile cells in hæmolymph gland of Sheep 3 after injection of hæmolymphocytotoxin. Mononuclear, polymorphonuclear, and polynuclear eosinophiles are found in the lymphadenoid tissue of the hæmolymph gland. Stained with hæmatoxylin and eosin.

Plate V.

Fig. 3.—Hæmatoidin in lymph gland of Sheep 3 after injection of hæmolymphocytotoxic serum. Stained with hæmatoxylin and eosin.

Fig. 4.—Iron-containing pigment found in spleen after injection of hæmolymphocytotoxin, demonstrated by the Prussian-blue reaction. Counter-stained with borax carmine.

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TABLE I.

Time.	Feb. 14, 10 A.M. Before injection.		Feb. 14, 2.30 P.M. 3½ hours after injection.		Feb. 14, 10.30 P.M.		Feb. 15, 8.30 A.M.		Feb. 15, 8.30 P.M.		Feb. 16, 8.30 A.M.		Feb. 16, 8.30 P.M.		Feb. 17, 8.30 A.M.	
	Per cent.	*Total.*	*Per cent.*	*Total*	*Per cent.*	*Total.*	*Per cent.*	*Total.*	*Per cent.*	*Total.*	*Per cent.*	*Total.*	*Per cent.*	*Total.*	*Per cent.*	*Total.*
·s].....	32.2	4,079	26.4	3,627	31.5	5,065	36.7	4,646	37.5	5,108	33.6	5,080	34.	4,821	30.8	4,035
clears.....	7.2	912	6.5	893	5.8	933	7.3	924	8.6	1,171	8.1	1,225	6.4	908	8.	1,048
.....	52.6	6,662	63.	8,656	58.7	9,439	52.1	6,596	44.1	6,006	52.1	7,878	51.8	7,345	52.	6,812
.....	8.	1,013	3.9	536	4.	643	3.7	469	9.8	1,335	6.	907	7.8	1,106	8.4	1,100
.....	0	0	.2	28	0	0	.2	25	0	0	.2	30	0	0	.8	105
cocytes.....	100	12,666	100	13,740	100	16,080	100	12,660	100	13,620	100	15,120	100	14,180	100	13,100
.....	7,500,000		7,000,000		7,500,000		7,000,000		6,800,000		6,800,000		7,200,000		7,200,000	

Differential count after injection of cold sterile physiological salt solution.

TABLE II.

	Dec. 1, 1 P.M. Before injection	Dec. 1, 7 P.M. 4 hrs. after injection.	Dec. 1, 12 P.M.	Dec. 2, 9 A.M.	Dec. 2, 4 P.M.	Dec. 3, 8 A.M.	Dec. 3, 8 P.M.	Dec. 4, 9 A.M.	Dec. 5, 9 A.M.	Dec. 6, 9 A.M.	Dec. 7, 9 A.M.
·s].....	4,735	6,504	4,791	5,137	4,833	7,023	7,058	7,156	5,256	5,558	4,725
clears.	410	537	285	475	1,076	1,045	919	1,097	546	854	824
.....	4,035	3,606	6,689	13,911	13,520	7,023	4,178	5,128	2,845	2,694	2,974
.....	140	86	95	178	117	1,209	1,338	1,364	754	894	919
.....	0	0	0	59	20	33	27	75	19	50	28
cocytes.	9,320	10,733	11,860	19,760	19,566	16,333	13,520	14,820	9,420	10,050	9,470
.....	12,000,000	12,800,000	13,340,000	14,240,000	13,640,000	13,620,000	13,640,000	13,380,000	14,100,000	13,200,000	14,520,000

Differential count after injection of normal serum of the goose (Sheep 2).

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TABLE XV.

	Mar. 25, 2 P.M. Before injection.	Mar. 25, 5.30 P.M. 3 hrs. after injection.	Mar. 25, 10.30 P.M.	Mar. 26, 8.30 A.M.	Mar. 26, 8.30 P.M.	Mar. 27, 8.30 A.M.	Mar. 27, 8.30 P.M.	Mar. 28, 8.30 A.M.	Mar. 29, 8.30 A.M.	Mar. 30, 8.30 A.M.	Mar. 31, 8.30 A.M.
...	2,857	1,491	1,470	2,300	2,699	2,491	2,242	3,067	2,767	4,235	2,965
ars.	379	136	124	289	305	202	235	270	262	494	349
Ti...	2,970	2,616	4,542	5,427	3,681	2,586	4,387	3,720	4,421	5,468	3,894
...	101	17	44	24	221	21	56	43	30	62	43
es... ...	13	0	0	0	14	0	0	0	0	21	15
onucles..	6,320	4,260	6,180	8,040	6,920	5,300	6,920	7,100	7,480	10,280	7,266
es.... ...	4,426,000	5,080,000	5,240,000	5,660,000	4,960,000	5,740,000	4,960,000	4,960,000	5,120,000	4,980,000	4,580,000

Differential count after injection of lymphotoxic serum (Sheep 11).

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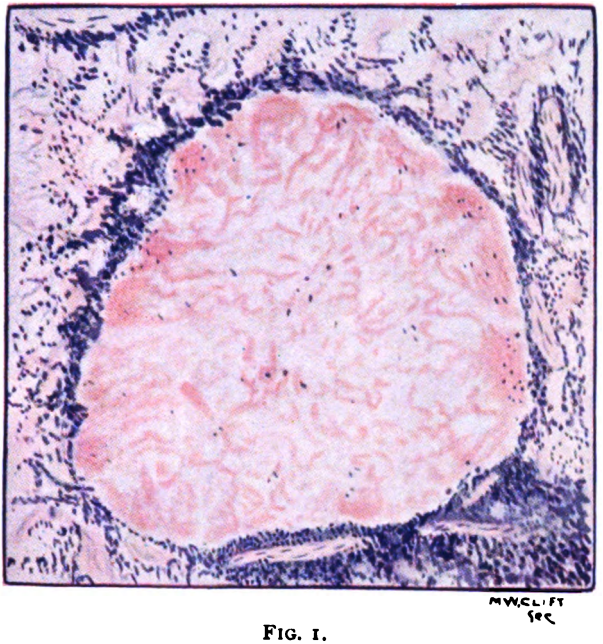

FIG. 1.

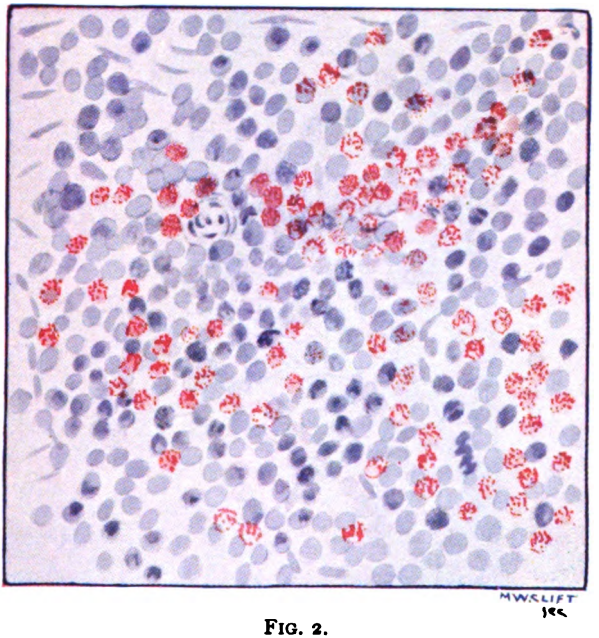

FIG. 2.

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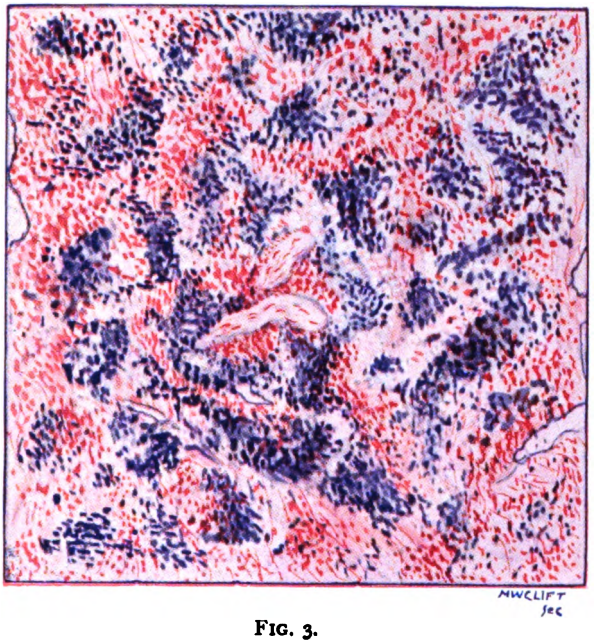

FIG. 3.

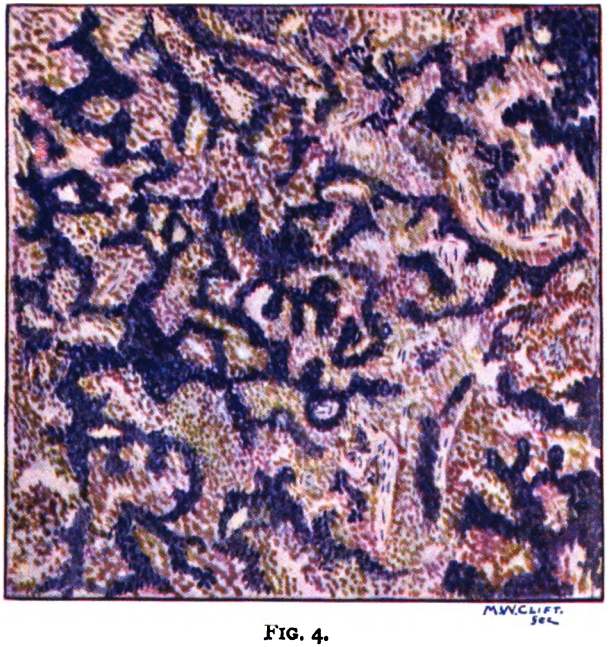

FIG. 4.

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STUDIES ON THE TOXICITY OF BILE.

I.

THE EFFECTS OF INTRAVENOUS INJECTIONS OF BILE UPON BLOOD PRESSURE.

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Plates XXIII–XXVI

In a recent communication by Arthur Edmunds,[1] it is stated that the fall of blood pressure produced by intravenous injection of bile salts is insignificant. The statement is supported by the reproduction of tracings obtained from such experiments. The one obtained after an injection of 5 cubic centimeters of a 1 per cent. solution of sodium glycocholate shows hardly any depression. The depression seen on the tracing obtained from an injection of 5 cubic centimeters of a 1 per cent. solution of sodium taurocholate corresponds to a fall of not more than 15 or 20 millimeters of mercury. The author states further that the subject was investigated in the same laboratory[2] a few years before by Dr. A. Young, who also found that the effect of bile salts on blood pressure was very slight. With 5 per cent. solutions of the salts the fall observed by him was hardly greater than that shown in the figures presented by the present author.

There have not been very many studies of the effect of bile upon the blood pressure, and of those made only few workers have given particulars or reproduced kymographic tracings. L. Traube,[3] who was the first one to study the blood-pressure effect of these salts by means of the kymograph, states that an intravenous

[1] Arthur Edmunds, *Brit. Med. Journal*, 1905, i, 57.

[2] Physiological Laboratory of King's College, London.

[3] L. Traube, *Berl. klin. Wochenschrift*, 1864, i, 85.

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injection of 4 to 6 cubic centimeters of a 33 per cent. solution of "Natrium choleinicum" produces a considerable lowering of the blood pressure, but he fails to give figures or to reproduce tracings presenting the character and the degree of the reduction of the pressure. M. Loewit,[4] who injected sodium cholate intravenously, states that the effect upon blood pressure is variable, depending upon the dose injected as well as upon the individuality of the animal. Besides Edmunds, Loewit is, as far as we know, the only one who published tracings presenting the lowering of the blood pressure, and the strongest effects which the latter author obtained were apparently not greater than those which were obtained by Edmunds. There are a few other writers who have reported the lowering effect of bile salts upon the blood pressure (Landois, Röhrig, Feltz and Ritter, and others), but their statements are not precise enough to permit a comparison of the degrees of the depressing effect. We have to add that nearly all the authors who studied the blood pressure confined their investigations exclusively to the effects of bile salts. There is nowhere any definite statement as to the effect of pure bile upon the blood pressure.

The results to be presented here were derived nearly exclusively from experiments made by intravenous injections of filtered ox bile or solutions of inspissated ox bile. We have chosen the whole bile in the first place for economic reasons, as taurocholic and glycocholic acids are too expensive for use in considerable quantities in a large number of experiments. But we wish also to record the statement that we have reason to doubt the validity of the now nearly universal belief that all the physiological and toxic effects of bile are due to the bile salts alone. It may be considered as proven that neither the bile pigments nor cholesterin nor any other constituent of the bile is capable of producing effects similar to those produced by the bile salts. But it is far from being proven for bile, as in fact it is not proven for any biological product, that the effect of the sum of all hitherto known parts is equal to the effect of the whole. Our doubts as to the absolute identification of the effects of bile with the bile salts

[4] M. Loewit, *Zeitschrift für Heilkunde*, 1881, ii, 459.

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arose in the course of another series of experiments on the toxicity of bile, and we shall therefore not enter here upon a discussion of the real merits of these doubts.

Our experiments were made on rabbits which were anæsthetized by ether. The injections of bile were made from a burette into the jugular vein and the blood pressure was taken from the carotid artery and transmitted by means of a mercury manometer to a rotating drum. The tubes connecting the artery with the manometer were filled with a solution of carbonate of soda or with a mixture of carbonate and bicarbonate of soda.[5] In some experiments tracheotomy was also performed so as to be in readiness for artificial respiration.

We may state at the outset that our experiments brought out often enough both extremes of action. Sometimes the injections of a good quantity of bile would exert only an "insignificant" influence upon the blood pressure, while at other times even a comparatively small dose of bile would produce a "considerable" fall of the pressure, and this occurred on the same animal and with the same concentration of the bile solution. However, these variations in the effects could be produced at will, for they depended largely upon the rate with which the bile was introduced into the circulation. We can therefore subscribe to the statement of Loewit that the dose of the injected bile exerts a distinct influence upon the degree of the effect, and admit also that the individuality of the animal may have something to do with the results. It might be added further that the concentration of the bile is a very important factor, and the general condition of the animal and the original height of the blood pressure, etc., are also factors in the reaction of the blood pressure to the introduction of bile. But all these factors together cannot compare with the importance of the rate of injection on the effect of the bile upon the blood pressure. Ten cubic centimeters and more of concentrated bile can be introduced into the veins of a rabbit without reducing the blood pressure by as much as 15 or 20 millimeters of mercury

[5] Under no circumstances should magnesium sulphate be used in the experiments. The reasons for this caution will be discussed in a future paper.

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if only the injection be carried out slowly. On the other hand, even 2 or 3 cubic centimeters of a moderately concentrated solution of bile are capable of reducing the blood pressure by 60 or 70 millimeters when it is permitted to flow rapidly into the vein.

Furthermore, the rapid injection of even a small quantity of bile may have a fatal effect upon the rabbit. Thus the question of the rate of injection may serve not only to reconcile the divergent statements with regard to the influence of bile upon blood pressure, but it may also clear up the contradictions which abound in the literature regarding the fatal effects of an intravenous injection, at least so far as the immediate effects are concerned.

In the early part of the last century it was stated by Magendie and other investigators that an intravenous injection of bile is invariably fatal to the animal. However, the bile which was used was not filtered and it was later assumed that the death of the animal was due to emboli produced by the impurities of the bile. Bouisson reported that rabbits which received 6 cubic centimeters of filtered bile survived the injections. During the last fifty years numerous experiments were carried out with clear solutions of biliary salts, free from corpuscular and from muciginous elements. In the literature on this aspect of the subject we nevertheless meet the same puzzling conflicting experiences. Some authors, such as Frerichs, for instance, have never seen any fatal results from the injection of bile except when air entered the veins. Others, like Leyden, Von Dusch, etc., lost at times some of their animals immediately after an injection of only 1 cubic centimeter of a 10 per cent. solution of bile salts. Von Dusch lost some of his rabbits two or three minutes after injecting a few cubic centimeters of filtered ox bile.

Judging from our experience we have reason to believe that all these contradictions have their chief origin in the variation of the rate with which the injections were made. Six to seven cubic centimeters of bile seem to have been the largest quantity which was ever injected into a rabbit. We have injected into

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one rabbit as much as 20 cubic centimeters of a 50 per cent. solution of ox bile within forty-four minutes, and not only was the rabbit apparently in a normal state at the conclusion of the injection but the circulation was even improved; the blood pressure having risen from 100 millimeters of mercury at the beginning to 118 millimeters at the end of the experiment. By extending the injection over a longer period, we have in another experiment injected 50 cubic centimeters of a 50 per cent. solution of ox bile without any visible immediate detriment to the animal. On the other hand, a small dose of 2 or 3 cubic centimeters of bile sufficed to kill a rabbit almost instantaneously when injected very rapidly. We have made, intentionally and unintentionally, a number of such experiments. The blood pressure would sink rapidly to so low a level that even immediately instituted artificial respiration failed to restore it. (Fig. 1 and Fig. 5, Plate XXIII.) The animals died frequently while under respiratory convulsions, which explains the statement of some writers that intravenous injections of bile often cause immediate convulsions.

That the rapidity with which the injections are carried out is an important factor in the results obtained is of course not a new idea. It has often been mentioned by writers dealing with the effects of injections of active substances, and was especially mentioned in connection with the recent studies on the toxicity of urine. But this factor has not been mentioned, at least as far as we know, by writers dealing with the effects of injection of bile upon the circulation. We missed especially allusion to it in the article of Edmunds, since in dealing there with the effects of potassium chloride the tracing given shows that the quantity of the salt which when injected within eight seconds caused only a moderate depression, caused when injected within two seconds a fatal fall of the blood pressure.

That the considerable fall of blood pressure which is caused by a rapid injection of bile is due to the toxicity of the bile and not to the mechanical effect of the injection we have proved by rapidly injecting a solution of 0.9 per cent. sodium chloride. An injection of 10 cubic centimeters or more of sodium chloride

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in 20 or 30 seconds marks hardly any change on the blood-pressure curve, while less than half this amount of bile injected within a similarly short time is surely fatal to the animal. In Fig. 2, Plate XXIII, at 1, 10 cubic centimeters of sodium chloride were injected in 10 seconds, and at 2, the same amount of the normal salt solution was injected within 20 seconds. In both cases there was scarcely any effect. Eight minutes later, 4.5 cubic centimeters of pure bile (at 3) were injected within 30 seconds, and the blood pressure sank rapidly, the animal dying within 3 minutes after the injection.

The same experiment also serves to demonstrate that the fall in blood pressure is not due to the low temperature of the bile, since the salt solution as well as the bile was injected at room temperature.

An analysis of our results brings out instructive details. The suddenness with which the blood pressure falls and the steepness of the descending branch in the blood-pressure curve depend essentially upon the rapidity of the injection. In rapid injections the fall of pressure sets in immediately at the beginning of the injection, and only exceptionally is it preceded by a very short slight rise (Fig. 2 at 3, Plate XXIII). The curve representing the fall is the steeper the more rapid the injection. If the injection was made more slowly either at the beginning or at the end, or at any phase, this fact appears well marked on the curve; and when after a considerable fall the injection was slowed, the pressure began to rise before the injection was finished (Fig. 4, Plate XXIII). In rapid non-fatal injections the blood pressure begins to rise immediately on cessation of the injection, and the ascending branch of the curve becomes for the most part a counterpart of the descending branch. In other words, the blood pressure rises at the same rate as it fell. Sometimes the pressure rises a little above the original level only to fall again immediately, for a short period, below this level. A momentary rapid rise to a point above the original height may occur even in cases of a fatal drop of blood pressure (Fig. 5, Plate XXIII).

If care be taken to avoid considerable depressions, rapid injections may often be repeated and kept up for a comparatively

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long period without affecting the mean level of the pressure; indeed in some experiments the blood pressure was at the end higher than at the beginning.

When the injection is carried on slowly there may at first be no effect at all. Later the respiratory, Traube-Herring, and other waves of the normal blood-pressure curve disappear one after another and the blood pressure commences to descend slowly. When a slow injection is extended over many minutes, the blood pressure may become reduced by 50 or more per cent. However, the curve representing the fall is not throughout a straight line; the more it descends the more it becomes concave at its upper aspect, which shows that the depressing effect of the bile is gradually diminishing (Fig. 3, Plate XXIV). Nevertheless, even with slow injections, if continued too long, a fatal level from which recovery is no longer possible may be reached. When a slow injection is interrupted, the beginning of the return of the pressure is sometimes delayed a little; but when the return begins the ascending branch of the curve here too is nearly a counterpart of the descending branch; it rises at first slowly and then more rapidly, and the last result, as a rule, is the return of the waves. In slow injections, however, the return is not entirely complete, and the oftener the injection is repeated the lower the level of mean pressure becomes, so that at the end of such an experiment, even if not very prolonged, the blood pressure may be much lower than at the beginning. However, this result is met with chiefly in those experiments in which the injection extends over longer periods of time.

While the rapidity of the fall thus depends essentially upon the rapidity of the injection, the degree of the fall is greatly influenced by the concentration of the bile. For instance, 1 cubic centimeter of a 2 per cent. solution of ox bile injected within ten seconds will reduce the pressure only by 10 to 12 millimeters (see Fig. 7, Plate XXIV), whereas 1 cubic centimeter of undiluted bile injected at the same rate will reduce the pressure by 30 millimeters and more (Fig. 3, Plate XXIV), and 1 cubic centimeter of a 20 per cent. solution of inspissated ox bile injected within 60 seconds will reduce the pressure by 50 or 60 milli-

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meters (Fig. 8, Plate XXIV), and the same solution injected in 20 seconds will surely be fatal to the animal (Figs. 1, 2, and 9, Plates XXIII and XXV).

These facts again show that the fall of blood pressure is due to the specific effects of the bile and not to mechanical causes or to the lower temperature of the injected fluids.

From a few observations we have made it would seem that the general condition of the animal and the original height of the blood pressure are factors also influencing the degree of fall produced by the injection of bile. Furthermore, in some cases the injection of bile caused immediately a slowing of the heart-beat, and in those cases the blood pressure reacted more readily and sometimes fell rapidly to a fatal level. In them the descending curve is very steep and shows only a few heart-beats (Figs. 1 and 5, Plate XXIII).

All writers agree that it is the detrimental effect of the bile upon the heart which causes the fall in the blood pressure, although there is disagreement as to whether the heart muscle, or the heart ganglia, or both, become affected by the bile. As regards the nature of the affection, Traube, Leyden, and others believe it to be malnutrition of the heart caused by the destructive action of the bile upon the blood corpuscles. There is no longer any doubt that bile is a strongly hæmolytic substance, but it is an open question whether this factor is especially active in causing the drop in blood pressure. Even at body temperature it takes some time for bile to produce perceptible hæmolysis, whereas the injection of bile into the circulation may produce a fatal drop of blood pressure in a few minutes. We have, however, disproven the hæmolytic theory by a direct experiment. By injecting rapidly a few cubic centimetres of bile, the blood pressure dropped quickly to a fatal level (Fig. 1, Plate XXIII) and the animal died in less than two minutes. The blood was immediately taken from the right ventricle and mixed with normal salt solution; no trace of hæmolysis could be discovered. It can be safely assumed, we believe, that hæmolysis has no share in the fall of blood pressure and the sudden death of the animal following immediately upon the injection of bile.

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The autopsies performed immediately after such sudden death have not shown definitely the cause of death. In one case there was considerable pulmonary œdema, the lungs were very hyperæmic, and the right ventricle contained a clot. In most of the autopsies, however, the findings were negative except that both ventricles were distinctly dilated and flabby. The heart's blood was either fluid or was firmly clotted in both ventricles. The lungs were normal. The appearance of the heart was suggestive of paralysis; and if this actually existed it may be conceived either as being due to anatomical changes in the organ or as being the expression of a functional effect, such as inhibition upon the heart's action. As regards the anatomical changes, it has been shown through various investigations that bile alters the structure of muscle tissue as well as nerve fibres and nerve cells. It would, however, be surprising should anatomical changes, as in the fatal cases on the one hand, take place within a few seconds from the beginning of the injection, and as in the case of non-fatal effects, such a rapid structural restitution on the other hand, occur.

Regarding the inhibitory effect of the bile upon the heart, we wish to report some interesting experiments. Before introducing the bile in certain rabbits, one vagus (left) was cut and the minimum strength of electrical stimulus, which caused a distinct inhibitory effect by application to the peripheral end, was established. For the induction coil we used one Daniell cell, and its distance was 300 and 250 millimeters in the rabbits in which this experiment was carried on. A dose of bile, which caused a considerable fall of the blood pressure, was injected, and during the persistence of the fall the irritability of the peripheral end of the vagus was tested, and it was found that not only had the irritability not suffered but that the effect was stronger than before the injection; moreover, in two instances a stimulus which was ineffective before the injection became effective during the fall of the blood pressure (Figs. 8, 9, 11, and especially Fig. 10, Plates XXIV, XXV, and XXVI). We are not aware that such experiments were made before.

In still other experiments we have made the following in-

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structive observations. Bile was introduced in such a manner as to cause a fatal fall of blood pressure and the peripheral end of the vagus was then stimulated. It was established that the inhibitory effect of the vagus remained intact as long as there were any signs of a heart-beat. Even when the pressure was not more than four millimeters and the heart-beat hardly perceptible, stimulation of the vagus caused a standstill of the heart (see especially Fig. 9, Plate XXV). O. Sokolow [6] and Loewit [7] state that vagus inhibition passes off before the heart stops. They have published no tracings and have given no data. Our tracings show that not only does the vagus inhibition last as long as the heart-beat, but that it retains its full irritability to the very end.

Our experiments further demonstrate that while the heart's action rapidly fails under the influence of bile, the inhibitory mechanism so far from being impaired by it may even be improved. This fact would favor the view that the effect of the bile upon the heart is a functional one, or, in other words, that bile stops the heart by inhibiting its activity. We do not mean that bile effects the inhibition through the vagus, because we know that the heart is stopped by bile even after eliminating the inhibitory effect of the vagus by a previous atropinization of the animal. Bile may, however, affect the heart in the same manner as potassium salts, the substances which contain three methyl ammonium groups, (Luchsinger and his pupils) and lactic acid (Gaskell [8]), which "weaken the contractions of the heart and ultimately bring it to a diastolic standstill." We see no reason why this action should not be called inhibition, brought about by some kind of a mechanism which is located in the muscle fibres or in invisible ganglia—whichever one chooses to assume. We may state further that we can see no objection to the assumption that the inhibition of the vagus is carried out through this mechanism and that atropin only interrupts the transmission of the vagus impulses through it. The latter

[6] Sokolow, *Physiol. u. toxicol. Studien am Herzen*, Inaug. Dissert., Bern, 1881.
[7] Loewit, *l. c.*
[8] Gaskell, *Schaefer's Text-Book of Physiology*, ii, p. 222.

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assumption would, however, collide with the prevailing orthodox views on the subject upon which we do not wish here to enter into a discussion.

DESCRIPTION OF PLATES.

The tracings were obtained in the usual manner by connecting the carotid artery with a mercury manometer which transmitted the variations of the blood pressure upon a revolving drum covered with smoked paper. The curves read from left to right. Besides the blood-pressure curve, there were recorded on each figure a base line, a time-marking line mostly in four seconds, and sometimes also a line marking the beginning and the end of an injection. For lack of space these lines were omitted in most of the reproduced figures. The bile used was either filtered ox bile, pure or dilute, or dissolved inspissated ox bile.

Fig. 1. 2.5 c. c. of 10 % ox bile rapidly injected into the jugular vein, the injection finished in 4 seconds. The blood pressure immediately dropped deep down and the animal was dead in less than 2 minutes. The blood from the right ventricle showed no hæmolysis.

Fig. 2. At "1" 10 c. c. of 0.9 % sodium chloride were injected in 20 seconds; at "2" the same amount of the same solution was injected within 10 seconds; both injections produced scarcely any perceptible effects upon the blood pressure. At "3" 4.5 c. c. of pure bile were injected in 30 seconds. There was at the beginning a very short slight rise, then a rapid fall of the blood pressure followed. The pulse became slower and artificial respiration was instituted, but the animal died in less than 3 minutes.

Fig. 3. At "1" 1 c. c. of undiluted bile was injected in 30 seconds; there was hardly a perceptible effect upon the pressure; at "2" and "3" each 1 c. c. more was injected in 9 seconds, the falls produced were equal to 25 and 30 millimeters of mercury; a slight rise preceded the fall. At the last point of the fall the injection was finished, and immediately the pressure rose again in the same manner as it fell. At x in "4" the injection of 5 c. c. of bile began and lasted 4 minutes; at x at the bottom of the curve the injection was finished, the low pressure lasting a few seconds, after which the rise began and reached the former level. The end of the curve is not reproduced.

Fig. 4. 2.5 c. c. of undiluted bile injected in 40 seconds. The injection was made at first slowly, then more rapidly, and finally considerably more slowly. The pressure began to rise before the injection was finished. The different rates of injection are well shown on the tracing.

Fig. 5. 2 c. c. of 50 % bile injected in 20 seconds. The pulse became very slow and the pressure fell very rapidly to a dangerous depth. At the end of the injection the pressure rose rapidly to above the original level, only to fall again immediately to a fatal level.

Fig. 6. At the arrow the slow injection of 5 c. c. of undiluted bile began and was finished at the cross at the bottom of the curve in 3 minutes. The

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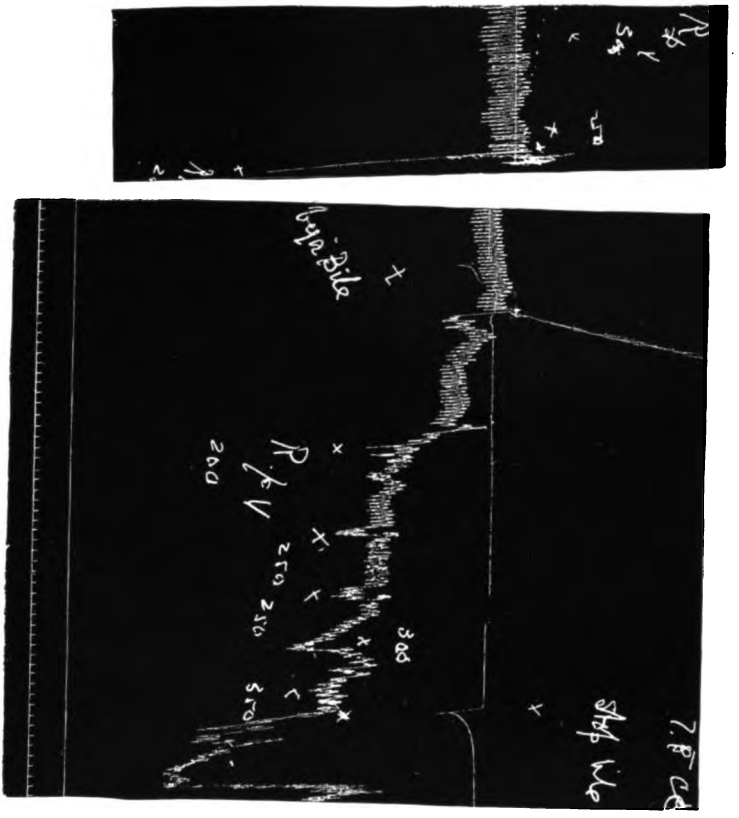

FIG. 10.

FIG. 11.

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[From THE JOHNS HOPKINS HOSPITAL BULLETIN, Vol. XVI, No. 166, January, 1905.]

THE EFFECT OF THE BILE UPON THE ESTER-SPLITTING ACTION OF PANCREATIC JUICE.[1]

A PRELIMINARY COMMUNICATION.

BY ALBION WALTER HEWLETT, M. D.

(From the Physiological Laboratory of the Cooper Medical College, San Francisco, Cal.)

That the bile assists in the digestion and absorption of fats [20] has long been recognized, but the manner in which it does this has never been fully worked out. The presence of bile favors the emulsification of fats and this indirectly aids the fat-splitting action of the pancreatic juice, because the finer the emulsion the greater is the surface of fat presented to the action of the fat-splitting enzyme. Little regard, however, has been paid to the possibility that the bile might assist the enzyme in any more direct manner. Pawlow[2] indeed asserts that the presence of bile so changes the medium that the fat-splitting proceeds two or three times as fast as it would if no bile were present. Bruno, a pupil of Pawlow, estimates the acceleration of the lipolytic action as ten to twenty times.[3] Glassner, working on pancreatic juice obtained from a human pancreatic fistula, found that the addition of bile to the pancreatic juice increased its action upon olive oil about one and one-half times.[4] The manner in which the bile assisted the pancreatic lipase was not especially considered by these investigators.

At the suggestion of Dr. W. E. Garrey, I have endeavored to ascertain more definitely the effect of bile upon the ester-splitting action of pure pancreatic secretion. The latter was

[1] This research has been completed by the aid of a grant from the Rockefeller Institute for Medical Research.

[2] Pawlow: The Work of the Digestive Glands. Trans. by W. H. Thompson, p. 159.

[3] Die Galle als wichtiges Agent bei der Verdauung. Inaug. Diss. St. Petersburg, 1897-98. Ref. in Jahresber. Thier-Chemie. Vol. 27, p. 441.

[4] Ztft. f. physiol. Chem., Vol. 40, p. 465.

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[20] obtained from dogs by means of secretin injections[1] or by injections of both secretin and pilocarpin. In this manner varying amounts, up to forty cubic centimeters, of pure pancreatic juice were obtained from each of a series of dogs and the action of this secretion was tested upon a variety of esters with and without the addition of bile.

Pure pancreatic juice decomposes ethyl butyrate surprisingly slowly if we compare it with the action of pancreatic extracts upon the same ester.[2] If one or two cubic centimeters of pancreatic juice be diluted up to five cubic centimeters with water and if the mixture be allowed to act upon 0.25 cc. of ethyl butyrate for twenty-four hours at 37° C., the difference between the acidity produced and that produced by an equal amount of boiled pancreatic juice is usually about 0.1-0.2 cc. of a 1/20 normal solution, although it may in some instances run up to 1.0 cc. or slightly more. Unboiled bile also decomposes ethyl butyrate very slowly, not differing appreciably in this respect from boiled bile. If the bile be added to the pancreatic juice, however, the cleavage of the ethyl butyrate proceeds with great rapidity and in twenty-four hours an acidity amounting to 7.0-16.0 cc. 1/20 normal solution will be produced. Similar results were obtained when ethyl acetate or amyl acetate were used in place of ethyl butyrate.

The ordinary fats are insoluble in water and they are therefore but little suited to a solution of the problem at hand, because any acceleration in the action of the pancreatic juice, which the bile would cause, might be attributed to its emulsifying action. This difficulty however, is, overcome if a soluble triglyceride, such as triacetin, be used, for then the question as to the degree of emulsification is eliminated. It was found that pure pancreatic juice, even without the addition of bile, splits triacetin much more readily than it does ethyl butyrate, ethyl acetate, or amyl acetate. In one experiment the acidity produced in an hour at 20° C. amounted to 0.5 cc. 1/20 normal solution and in twenty-four hours to 12.6 cc. On the addition of bile, the decomposition proceeded at a much more rapid rate just as it did in the case of ethyl

[1] Bayliss and Starling: The Mechanism of Pancreatic Secretion. Jour. of Physiol., Vol. 28, p. 325.

[2] Kastle and Loevenhart: Am. Chem. Jour., Vol. 24, p. 491.

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butyrate. The acidity produced in one hour amounted to [20]
13.0 cc. and in twenty-four hours to 18.6 cc. The acceleration, therefore, was very marked during the first hour, and the reason that this acceleration was less apparent after twenty-four hours probably lay in the fact that the reaction was approaching an equilibrium.

The addition of bile also accelerates the action of the pancreatic secretion upon emulsions of olive oil, but to what extent and why this occurs has not as yet been fully determined.

Which constituent of the bile possesses this peculiar property of accelerating the ester-splitting action of the pancreatic juice? Boiling the bile does not destroy this property and we may therefore infer that it is not due to an enzyme. Furthermore, this property resides neither in the cholesterin, in the pigments, in variations in reaction, nor in variations in the amount of calcium salts present. Precisely the same accelerating effects may, however, be produced by the addition of lecithin to the pancreatic juice. Thus in one experiment the pure pancreatic juice by its action on triacetin for twenty-four hours produced an acidity amounting to 4.3 cc. 1/20 normal solution; the same plus two cubic centimeters of bile produced 19.5 cc. acidity, and plus two drops of a strong alcoholic solution of Merck's lecithin produced an acidity of 19.9 cc. A commercial preparation of the bile salts will also accelerate the ester-splitting of the pancreatic juice, but it seems that the more the bile salts are purified, the less effect they have in this respect, so that it is possible that the action of the crude preparation is due to a con- [21]
tamination with lecithin.

Various possibilities present themselves as to the manner in which the bile assists the ester-splitting action of the pancreatic juice. In the first place, does it act by converting a proferment into a ferment, an action analogous to that exerted by the enterokinase of the intestines upon the proferment of trypsin?[1] In considering this question, we must remember that if this hypothesis were true, a small amount of bile would be just as effective as a larger amount provided

[1] Bayliss and Starling: The Proteolytic Activities of the Pancreatic Juice. Jour. of Physiol., Vol. 30, p. 61.

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[21] it acted for a sufficiently long time, and that if sufficiently small amounts of bile were added to the pancreatic juice the rate of cleavage could be shown to progressively increase as more and more of the proferment were converted into the ferment. It was found as a matter of fact that these conditions are not realized in the case of the action of bile upon the pancreatic juice. Another possibility is that there is a substance in the pancreatic juice, itself inert, which combines with some constituent in the bile to form the fat-splitting enzyme. Such a condition would be analogous to that described by Kyes* for cobra venom. Alone this exerts little if any hæmolytic action; plus lecithin the action is most marked. The pancreatic juice, however, is moderately active even without the addition of bile. It would, therefore, be necessary to suppose that the pancreatic juice normally contains a trace of lecithin, but that this is not sufficient to render active any considerable portion of the fat-splitting ferment. It seems to us more probable that the bile acts as an accelerator upon the fat-splitting ferment, and indeed this is more in accord with the modern view that all ferment action is in the nature of an acceleration. We know that the esters will slowly decompose in pure water and the same is true if they are placed in boiled pancreatic juice. If the secretion be unboiled, *i. e.*, if the ferment be present, the decomposition proceeds at a somewhat more rapid rate, and if to this, bile be added, the decomposition is still further accelerated. We do not know exactly how this last acceleration is brought about, although other similar accelerations are known to us. Thus hydrogen dioxide will slowly decompose in water. If ferric sulphate be added to the water, the decomposition is moderately accelerated. Cupric sulphate alone produces little if any effect upon the hydrogen dioxide, but if it be added to the mixture of hydrogen dioxide and ferric sulphate the reaction is enormously accelerated. Substances which act in this manner have been termed zymoexcitors. It seems probable, therefore, that the bile increases the ester-splitting action of the pancreatic juice by virtue of a zymoexcitor which it contains and that this zymoexcitor is in part, at least, lecithin.

* Berl. klin. Wochenschr., 1902, Nos. 38-39.

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A STUDY OF THE DEJECTA OF NORMAL CHILDREN AND OF THOSE SUFFERING FROM ACUTE AND SUBACUTE DIARRHEA WITH REFERENCE TO B. DYSENTERIAE

KATHARINE R. COLLINS

(From The Rockefeller Institute of Medical Research and the Research Laboratory of the Department of Health, New York City)

Reprinted from

THE JOURNAL OF INFECTIOUS DISEASES, Vol. II, No. 4, November 25, 1905, pp. 620–626

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A STUDY OF THE DEJECTA OF NORMAL CHILDREN AND OF THOSE SUFFERING FROM ACUTE AND SUBACUTE DIARRHEA WITH REFERENCE TO B. DYSENTERIAE.*

KATHARINE R. COLLINS.

(From The Rockefeller Institute of Medical Research and the Research Laboratory of the Department of Health, New York City.)

UP to the present time, except in a limited number of cases, B. dysenteriae, though widely searched for, has not been found in the dejecta of normal individuals or those suffering from diseases other than dysentery.

Shiga,[1] Flexner,[2] Drigalski,[3] and others, after the examination of a large number of cases, chiefly adults, failed to demonstrate the presence of this organism in normal stools.

Duval[4] states that he found in two instances B. paradysenteriae of the Flexner-Harris type in the normal stools of milk fed infants.

Wollstein[5] in a series of 32 non-dysenteric infants, did not find B. dysenteriae in any instance. In 24 other cases examined at autopsy, B. paradysenteriae was found in the scrapings from the mucous membrane of the intestines three times. Two of these cases gave a history of previous attacks of diarrhea, while the third case had developed a terminal dysentery five days before death.

The term diarrhea is used wherever the exact nature of the attack was not ascertainable; while the term dysentery is used only in those cases where the organism was isolated. The term paradysentery is applied to the types which ferment mannite and differ in their agglutination reaction, but in all other respects correspond to the organism isolated by Shiga as the cause of dysentery.

* Received for publication August 23, 1905.

1 *Centralbl. f. Bakt.*, 1898, 23, p. 599. *Ibid.*, 1898, 24, pp. 817, 870, 913. *Deutsche med Woch.*, 1901, 27, pp. 741, 765, 783.

2 *Studies from The Rockefeller Inst. of Med. Res.*, 1904, 2, p. 121.

3 *Veröff. a. j. mil. Sanitätswesen*, 1902, 20, p. 86.

4 *Studies from The Rockefeller Inst. of Med. Res.*, 1904, 2, p. 42.

5 *Ibid.*, p. 193.

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Charlton and Jehle,[1] working in the laboratory of St. Ann's Kinderhospital, Vienna, found, in apparently normal stools, B. paradysenteriae, Flexner-Manila type, present in two cases out of ten examined. These cases gave a negative history as to dysentery and diarrhea. The blood of the cases did not agglutinate the cultures obtained from the stools or the Flexner-Manila bacillus.

The following report comprises a study of 78 cases, of which 57 were children with normal stools and 21 were children suffering from acute or subacute diarrhea.

The first series of cases was undertaken during the months of March and April, 1903. At this time, a mild epidemic of dysentery existed in some of the wards at the Nursery and Child's Hospital, New York City. From the stools of several of these cases B. paradysenteriae, Park-Mt. Desert type, was isolated. In one case the organism was obtained from the stools before death and at autopsy from the mucous membrane of the lower colon. Thirteen infants having normal stools were selected from these infected wards for examination. In every instance the result was negative.

No special method was used to obtain the material for examination, plates being made as early as possible from the stools as normally passed.

During the month of August of the same year, coincident with the existence of dysentery in some of the wards, the study of normal stools was resumed. On this occasion the cases were taken from the reception ward where dysentery was, presumably, not present. The children examined were for the most part nurselings.

Two cases had had dysentery a few weeks previous to this time; from one case B. paradysenteriae had been isolated. At the time of the examination, both infants had recovered and the stools were normal. In neither case was B. paradysenteriae found. Seven of the remaining ten cases were also negative, while the tenth case was positive, giving the following history:

Case 1.—Age 24 months, nurseling, healthy. Had been in the reception ward three weeks; during this time the stools were normal and there was

[1] *Trans. Assn. American Phys.*, 1904, 19, p. 405.

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no history of diarrhea before entering the hospital. The child developed pneumonia three days after the stools were examined and the organism found. In the beginning of the attack there was a slight amount of mucus, but in every other respect the stools were normal and remained so throughout the course of the disease. The blood gave a good reaction in dilution of 1:10 with the Flexner-Manila organism.

The organism isolated in this case in the proportion of one out of one hundred colonies possesses the following characteristics: Non-motile bacillus, ferments mannite and maltose without production of gas, does not produce gas on glucose or lactose, produces indol and nitrites, renders litmus milk slightly acid which later becomes alkaline. In the above features it corresponds to the Flexner-Manila type. In its agglutination reactions it resembles the Coney Island and Salant organisms which were isolated from cases of typical clinical dysentery.

A polyvalent dysenteric horse serum agglutinates this bacillus completely in the dilution of 1:500. It raises specific agglutinins in the animal body for itself and common agglutinins for other mannite fermenting types of the dysentery organism. The following table shows by the absorption tests its relation to the Coney Island and Salant cultures:

TABLE 1.

SERUM OF A HORSE INOCULATED WITH SHIGA, FLEXNER-MANILA, AND PARK-MT. DESERT TYPES OF B. DYSENTERIAE EXHAUSTED WITH THE CONEY ISLAND AND MT. DESERT TYPES.

Cultures	Serum before Exhaustion						Serum after Exhaustion with Coney Island Type						Serum after Exhaustion with Mt. Desert Type					
	20	50	100	200	500	1000	20	50	100	200	500	1000	20	50	100	200	500	1000
Japan—Shiga.....	++	++	++	++	++	++	++	++	++	++	++	−	++	++	++	++		
Manila—Flexner.....	++	++	++	++	++	++	++	++	++	++	++	−	++	++	++	++		
Mt. Desert—Park.....	−	++	++	++	++	++	++	1					−	−				
Coney Island—Collins	++	++	++	++			−						++	++	−			
New York—Salant....	++	++	++	++			−						++	++	−			
Normal—Collins.....	++	++	++	++			−						++	++	−			

++ Complete agglutination, 1 tendency, − negative.

In the above table the three last cultures exhibit a similar reaction after exhaustion. It is of interest to note that the production of common agglutinins for the Coney Island type is much less than for the Mt. Desert type.

In the above series of examinations the material was obtained by irrigation of the lower colon with sterile water. Plates were made from the first and last parts of the washing. From the

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latter, bits of mucus could readily be picked out. After withdrawal of the tube, the rectum was swabbed with sterile cotton and plates were made also from the swab.

The next study of 54 cases, obtained from several different sources, was made during the months of July, August, and September, 1904. Thirty-three of these cases were children with normal stools, and 21 were children suffering from acute or subacute diarrhea. In obtaining material for examination in these cases, I used the method devised by Dr. William H. Park. This consists of a blind glass tube with quite a good sized opening on the side. The tube is inserted into the rectum and the mucous surface, exposed through the opening in the side, is washed with sterile water. A cotton swab is then passed several times over the surface of the membrane; the swab is placed in a tube of broth and plates are made from this. A platinum loop was tried for scraping the mucous membrane, but it offered no advantage over the swab and was more irritating. In some cases irrigation of the lower colon supplemented the use of the tube, but as the use of the latter eliminated, to a very great extent, the gas producing organisms, it proved a more satisfactory method.

Of the normal cases examined, 17 were obtained through the courtesy of Dr. Reed from the Babies' Hospital, New York City. These were bottle fed babies ranging in age from three weeks to 21 months. Dysentery existed in the wards at this time, and some gave a past history of diarrhea, but, at the time of the examination, the stools were normal. In none of these 17 cases was B. dysenteriae or paradysenteriae found to be present.

Thirteen normal cases taken from the Foundling Hospital, New York City, were children ranging from two and a half to seven years of age, who had been in the wards where dysentery existed from three weeks to three years. Out of the 13 cases two were positive, the history being as follows:

Case 2.—Age 7 years. Eczema. Had been in the ward three years, and during that time there was no history of diarrhea. The stools were normal at the time of examination. The blood of the child reacted fairly in dilution of 1:20 with the Mt. Desert type of organism, but failed to show even a trace with the Shiga or Flexner-Manila type.

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From the mucous surface of the rectum B. paradysenteriae, Mt. Desert type, was isolated. This organism agglutinated with a polyvalent dysenteric horse serum in the dilution of 1:500. With the Mt. Desert specific serum,* it agglutinated in the dilution of 1:200, which was the full agglutinating index of the serum, but failed to agglutinate with the Flexner-Manila or Shiga specific serum.

Case 3.—Age 3 years. Chronic conjunctivitis. One year in the ward, during which time there was no history of diarrhea. The stools were normal at the time. The blood in this case reacted feebly in dilution of 1:20 with the Flexner-Manila organism and not at all with the other types.

From the rectal mucous membrane B. paradysenteriae, Flexner-Manila type, was isolated in proportion of 1:50 of the colonies fished. It agglutinated with the polyvalent serum in the dilution of 1:500; with Flexner-Manila specific 1:100, and was negative with the specific sera of the other types.

During the past summer an epidemic of dysentery occurred in several districts, widely separated from each other, on Staten Island. Through the kindness of Dr. Walser and Dr. Patterson of Staten Island, the blood and stools from several of the dysenteric patients were obtained for examination and, as far as observed, only the Shiga type of organism prevailed.

From one of these infected neighborhoods where the sanitary surroundings were very poor three normal cases were taken under the following conditions:

Case A.—Age 7 years. No history of diarrhea. Three members of the family had had dysentery of the Shiga type. One case, in a child of three years, existed at the time of examination of the normal child, one adult case occurred three weeks previous, and one case two weeks later. Examination of the stools of the normal case was negative.

Cases B and C were taken from two children, two and five years of age, living directly oppposite to Case A. One child in this family, three years of age, had dysentery, Shiga type, at the time. The stools of the two normal children were negative.

The 21 cases of acute and subacute diarrhea were derived from the Babies' Hospital and the dispensaries of Bellevue Hospital and the German Polyclinic. The infants were bottle

*By specific serum is meant a polyvalent dysenteric serum from which all agglutinins have been absorbed except those for the one type.

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fed and were from six to eighteen months of age. The acute cases presented, in general, the following symptoms:

The onset was sudden, with vomiting, rise of temperature, which was generally high, stools frequent, thin and watery in appearance and sometimes green and frothy. The prostration was marked and mortality high.

In the subacute cases the vomiting was absent, the rise of temperature moderate, if any, the stools thin and sometimes green, with some mucus but no blood; the emaciation was great.

In these cases, the contents of the colon were examined as well as the mucous surface of the rectum, and in no instance was B. dysenteriae found.

Charlton and Jehle[1] did not find the dysentery bacillus in the stools of children suffering from so called summer diarrhea, and Weaver[2] also failed to demonstrate it as the cause of the summer diarrhea of children.

The limited number of cases reported in which B. dysenteriae has been found in the stools of healthy individuals, or those suffering from disease other than dysentery, together with the fact that thus far the organism has not been found normally in adults, renders the interpretation of this phase of the subject difficult.

Flexner[3] has pointed out the advisability of questioning the nature of this organism as to its purely parasitic existence. Investigations along this line will aid greatly in determining the significance of the presence of the organism in the normal individual and in cases where the definite clinical symptoms of dysentery are absent.

Reasoning from analogy, we would readily assume that B. dysenteriae, like B. diphtheriae and the pneumococcus, may lead a harmless existence in the normal individual until a lowered vitality of the tissues invaded offers a suitable soil for the full development and elaboration of its toxines.

Wollstein[4] calls attention to the difficulty of obtaining reliable histories in the cases of children. This seems to be the experience generally of those who have to do with children. Very few

[1] *Loc. cit.* [2] *Jour. Inf. Dis.*, 1905, 2, p. 81. [3] *Loc. cit.* [4] *Loc. cit.*

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infants, especially of the class generally found in dispensaries and hospitals, escape diarrhea in some form in the early months of life. Hence the finding of the organism under such conditions should not lead us into drawing too definite conclusions.

The failure to find B. dysenteriae or paradysenteriae in infants suffering from so called acute and subacute summer diarrhea, even though the symptoms were severe and repeated examinations were made, would lead us to suspect some cause or combination of causes other than this organism as the etiological factor in these conditions.

I desire to express my gratitude to Dr. William H. Park, Director of the Research Laboratory of the Department of Health, New York City, and to Dr. Simon Flexner, Director of The Rockefeller Institute for Medical Research, for their suggestions and criticisms of this work.

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Reprinted from the American Journal of Physiology.
VOL. XIV. — OCTOBER 2, 1905, — No. IV.

PHYSIOLOGICAL AND PHARMACOLOGICAL STUDIES OF MAGNESIUM SALTS. — I. GENERAL ANÆSTHESIA BY SUBCUTANEOUS INJECTIONS.

BY S. J. MELTZER AND JOHN AUER.
[*From the Rockefeller Institute for Medical Research.*]

INTRODUCTION.

THE salts of sodium, potassium, calcium, and magnesium are the chief inorganic compounds present in the tissues and fluids of the animal body. As to the rôle which these elements might play in the manifestations of life phenomena, the first three have been the subject of a vast number of studies in experimental physiology; magnesium, however, received hardly any attention. Wherever calcium is found in the body magnesium is also present. Recently calcium has received considerable attention. While there is as yet no unanimity of opinion concerning the actual part which calcium takes in the activities of the living body, all agree that it is an important factor in the mechanisms which are at the bottom of these activities. Should magnesium be no active factor in these mechanisms? Should magnesium, for instance, have no active share in the irritability of nerve and muscle tissues? This would be the more surprising since in muscle and nerve tissues magnesium is present in larger proportion than calcium, and it therefore seems that the affinity of those tissues is greater for magnesium than for calcium. This view is strengthened by feeding experiments, at least as far as muscle is concerned. When animals are fed magnesium compounds, that element is found greatly increased in the ash of muscles, while the percentage of magnesium in bone is but slightly greater than under a normal diet.[1] As nerve cells, nerve fibres, and muscle fibres are the most irritable, the most active tissues of the body, we should expect magnesium to take some essential part in the activities of the body, at least in the specific activities of the tissues mentioned.

[1] KÖNIG: Zeitschrift für Biologie, 1874, x, p. 69.

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However, the share which magnesium might be taking in the specific manifestations of nerve and muscle tissues need not be of the character which increases its specific activities. It is possible that the presence of magnesium is not at all for the purpose of increasing the specific energies of muscle and nerve, of favoring the contractility of muscle fibres, or of facilitating the conductivity of the nerve fibres, or the receptability and conductivity of nerve cells; on the contrary, the task of magnesium might be to render assistance in restricting, reducing, or inhibiting all the specific energies of these tissues. At a certain phase of functional activity the inhibition of a contraction of a muscle, or the inhibition of a reflex action, or of a sensation within a ganglion cell, is an indispensable factor in the prompt working of these functions, and these inhibitions require as much vital assistance for their development as is required and received by the positive energies of these tissues. The diastolic part in the cycle of a heart beat; the inhibition of the contraction of the inspiratory muscles in the cycle of a respiration; the inhibition of the disturbing activity of any antagonistic muscle; the inhibition of a disturbing concomitant reflex; the inhibition of the activity of one sense during the act of perception of another sense, — in these and in many other instances the suppression, the inhibition of an activity is an indispensable part of a vital function. Is it not possible that the processes of inhibition are essentially facilitated by compounds which contain magnesium?

This supposition suggested itself to us by an observation which one of us (M.) made a few years ago. In studying the effect of intracerebral injections it was found that magnesium sulphate acted differently from some of the other inorganic salts. In a paper[1] read at the meeting of the American Physiological Society in December, 1899, the effect of an intracerebral injection of potassium chlorate was described as producing a long series of convulsions, forced movements, opisthotonus, etc. The reported proceedings of this meeting contain the following passage: "The author demonstrated on a rabbit the described effects of potassium chlorate, and also on another rabbit, the *opposite effect of intracerebral injection of magnesium sulphate: without preceding convulsions the rabbit became paralyzed in a short time.*" From this observation it seemed that the primary effect of magnesium upon nerve cells is that of paralysis without any preceding excitation. At that time no comment was made by the author

[1] MELTZER: This journal, 1900, iii, p. ix.

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upon the demonstrated phenomenon; it was reserved for a further experimental analysis.

In the past year a number of experimental studies were made by us on the effect of magnesium salts upon the activity and irritability of the nervous system. The studies brought out some new facts which will be reported in several communications. In the present communication we shall deal with some effects of subcutaneous injections of magnesium salts. We may state at the outset, in a general way, that the several series of experiments we have carried on so far with magnesium salts seem indeed to confirm our original expectations: 1. Magnesium exerts a profound effect upon the nervous system. 2. The effect seems to be exclusively of an inhibitory character; we had so far not a single instance of an increase of excitability brought on by a magnesium salt.

However, in describing our several results we shall lay more stress upon the facts which our investigations bring to light than upon any general theory which our experiments may tend to prove.

Subcutaneous Injections of Magnesium Salts.

As we have already stated above, and as the sub-title indicates, we shall report in the present communication the observations made in the series of experiments with subcutaneous injections of magnesium salts. Such experiments have been made before. They were undertaken, however, from another point of view, and the results which they brought out were different from those we are able to communicate. In the seventies of the last century many experiments were made with subcutaneous injections of small doses of magnesium sulphate. The purpose of these experiments was to verify the statements made by Luton[1] and by Vulpian that injections of small doses of that salt produce purgation. Recke[2] reported later that subcutaneous injections of large doses of magnesium sulphate can be fatal to the animal. The same was claimed by Hay,[3] who stated that death occurs by paralysis of respiration. Mickwitz[4] claimed that magnesium chloride stops the heart before it can affect the respiration. Clessin[5] also studied

[1] Luton: Gazette hébdomadaire de médecine et de chirurgie, 1874, xxviii, p. 455.

[2] Recke: Dissertation, Göttingen, 1881.

[3] Hay: Journal of anatomy and physiology, 1883, xvii, p. 512.

[4] Mickwitz: Dissertation, Dorpat, 1874.

[5] Clessin: Dissertation, Würzburg, 1890.

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the toxic effect of chloride of magnesium upon the heart and muscles of the frog.

We employed in all of our experiments comparatively large doses of the salts, which gave indeed fatal results in some cases, and which we shall also describe. However, the effect which we have studied chiefly, and which was never studied or described before, is the production of anæsthesia by these salts. As our protocols will show, we obtained complete anæsthesia with subsequent full recovery in dogs, cats, rabbits, guinea-pigs, white rats, frogs, and fowls. For most of our experiments we have employed magnesium sulphate in 25 per cent solution. We have however established by a number of experiments that anæsthesia can also be produced promptly by magnesium chloride, and in a few tests we obtained similar results with magnesium bromide. We shall present our results by giving a few selected protocols from each series of experiments.

SUBCUTANEOUS INJECTIONS OF MAGNESIUM SULPHATE IN RABBITS.

Experiment 1. April 13. — Gray rabbit, female, 1500 gm., very active and restless; 120 respirations to the minute.

3 17 P.M. Injected subcutaneously into left upper flank 8 c.c. of a 25 per cent solution of magnesium sulphate.

3.20. Jumps off table, moving about floor in lively fashion, kept there.

3.30. Losing interest in surroundings.

3.40. Remains quietly in centre of room when put there; when forced to hop, hind legs are moved clumsily.

3.50. Practically unable to move; hind legs weaker than front legs.

3.51. Can be placed lying on side; front leg when placed behind ear remains there. Limbs perfectly flaccid, ears on back, chin on table. Limp as a rag when picked up. Probe in nasal cavity provokes only moderate resistance; pinching tail arouses animal somewhat.

3.59. 68 respirations to the minute.

4.00. Conjunctival reflex abolished; is not aroused by a noise close to the ears, but withdraws head slightly when probe is inserted into nose. Remains in practically the same condition for about two hours.

6.00. Animal recovered.

April 14. — Animal lively as ever; had no diarrhœa.

This animal received a subcutaneous injection of magnesium sulphate, 1.3 gm. per kilo, and was not massaged. About forty minutes after the injection the rabbit was apparently in a state of

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anæsthesia; every muscle was perfectly relaxed and the conjunctival reflex was gone. After remaining in that state for about two hours it recovered completely.

Experiment 2. May 2. — Gray rabbit, male (201), 1640 gm.; 64 respirations to the minute.

3.30 P.M. Received a subcutaneous injection of 10 c.c. of a 25 per cent solution of magnesium sulphate in right back. About 1 c.c. escaped. Place massaged.

4.05. Lying on side, unable to get up, front leg kept behind ear, conjunctival reflex very sluggish.

4.12. Held up by legs, no struggle, limp as a rag; no conjunctival reflex; no response to pinching tail.

4.30. Respiration 64 to the minute. Condition unchanged.

5.00. Got on his feet.

May 3. — Animal very active.

This animal received perhaps a little more magnesium sulphate than the previous one, and the place of injection was massaged. The anæsthesia, which also set in about forty minutes after injection, reached here a complete stage, but lasted hardly an hour.

Experiment 3. May 3. — White rabbit, female, 1420 gm.; 200 respirations to the minute.

4.07. Injected subcutaneously into right flank 12 c.c. of a 25 per cent solution of magnesium sulphate; massaged.

4.16. Animal sits quietly, weak and shaky when moving.

4.20. Lying motionless on side, breathing slow and very shallow.

4.22. Pupils dilated. Often weak kicks of hind legs, perfunctory convulsions from asphyxia; artificial respiration instituted.

4.26. Heart stopped beating.

This animal received 2 gm. per kilo, and the place of injection was massaged. Muscular weakness developed very rapidly, the respiration became very shallow, and fifteen minutes after injection complete asphyxia set in, but with only very slight indications of convulsions.

Experiment 4. May 4. — Gray rabbit, female, 1430 gm. (204, survivor from a previous subcutaneous injection). 76 respirations to the minute.

3.53. Injected subcutaneously into left flank 9 c.c. of a 25 per cent solution of magnesium sulphate; massaged.

4.06. Just about able to move; 128 respirations to the minute.

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4.15. Lies flat on side as placed.

4.37. No conjunctival reflex; animal limp as a rag, perfectly flaccid.

5.12. 52 respirations to the minute; inspiratory pause and active expiration.

6.05. Still flaccid, occasional slight movements of legs.

6.15. Got feet under body. Observation discontinued. Put into cage.

May 5. — Animal normal, had no diarrhœa.

This animal received 1.5 gm. magnesium sulphate per kilo, and was massaged. The anæsthesia was most complete; it set in early and lasted over two hours. The rabbit survived two injections without any after effect.

Experiment 5. — Gray rabbit, male, 1700 gm.; 160 respirations to the minute.

2.53. Injected subcutaneously into right flank 12 c.c. of a 25 per cent solution of magnesium sulphate.

3.22. Animal lying limp on back, no resistance when raised by leg.

Animal operated without any other anæsthesia. Cannulæ introduced into trachea, left carotid artery, and left pleural cavity; left vagus cut. Operations completed at 4.04. There was no struggle whatsoever, and no sign of pain throughout the operation.

Until 5.30 tracings of blood pressure and respiration were taken, and the central and peripheral ends of vagus often stimulated with induction currents (see Figs. 1 and 2). The blood pressure taken about 75 minutes after the injection of the magnesium salt amounted to nearly 100 mm. mercury; one hour later it fell to about 75 mm. Stimulation of the central end of the left vagus, which produced a strong effect upon the respiration, brought out no sign of pain. The blood in the carotid artery looked dark, although the respiration was good.

5.30. Trachea clamped: asphyxia brought out convulsions. Autopsy. Lively peristalsis of small intestines, which contain liquid material and gas. Bladder contains 30 c.c. of urine, which shows a powerful reduction with Trommer's test for sugar; and with sodium hydrate and Lugol's solution a strong odor of iodoform was produced (aceton).

In this experiment the animal received 1.75 gm. magnesium sulphate per kilo. It had a most perfect narcosis in about thirty minutes. A very extensive operation and usually painful stimulations could be carried on for nearly two hours without any sign of a pain reaction. Nevertheless the blood pressure, at least for the first part of the experiment, was nearly normal. Judging from the effect of stimulation of the central end of the vagus upon the respiration, the medulla oblongata was apparently still very sensitive. Had we in-

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terrupted the experiment and sewed up the wound, the animal probably would have survived the injection, operation, and experiment,

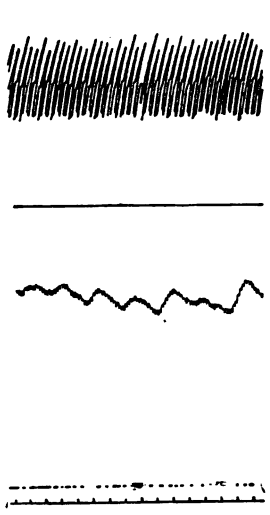

FIGURE 1. — One-half the original size. Blood pressure and respiration tracing from a rabbit (Experiment 5) seventy-five minutes after receiving a subcutaneous injection of magnesium sulphate, 1.75 gm. per kilo. Blood pressure was obtained with a mercury manometer, the fluid within the connecting tube being a mixture of sodium carbonate with hydrated sodium carbonate. The respiration was written by means of Meltzer's pleural cannula (see Zeitschrift für Instrumentenkunde, December, 1894). The upper tracing represents the respiration, upstroke the inspiration; the straight line below it marks the atmospheric pressure, and the space between corresponds to the negative pressure during expiration. The next wavy tracing represents blood pressure; the straight line below it is the 0-line for blood pressure. The lowest line marks the time in two-second intervals for both tracings, which were taken simultaneously as they are presented in this figure.

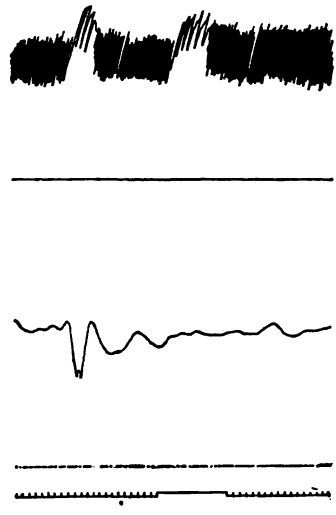

FIGURE 2. — One-half the original size. From the same animal as in Fig. 1, taken sixty minutes later. Tracings and lines the same as in Fig. 1, with a slower drum. The respiration curve shows the effect of stimulations with induction currents of the central end of the cut left vagus (250 and 200 mm. distances between the coils). The blood pressure curve shows the effect of a stimulation (200 mm. distance) of the peripheral cut end of the vagus upon the heart. The blood in the cannula began to clot, hence the small excursions.

regaining his normal state, — an occurrence we actually observed in other somewhat similar experiments.

These few abbreviated protocols illustrate sufficiently the essential results which we obtained in the series of experiments with subcuta-

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neous injections of magnesium sulphate in rabbits. With a certain dose of this salt we could obtain a complete anæsthesia lasting as long as two hours and even longer, from which the animals recovered without any perceptible after-effect. During this state of anæsthesia any operation could be performed and nerves stimulated without causing any pain or waking the animal. The muscles are completely relaxed. We may add here that direct and indirect stimulations of the muscles brought out prompt reactions. In some experiments the abdomen was opened during the anæsthesia without there being the slightest attempt to press the intestines out of the cavity. The conjunctival reflex was completely abolished; a probe in the nose would be tolerated without any reaction. (In this connection we have, however, to mention an instructive observation. Even when the animal was under such deep anæsthesia as not to react to the presence of a probe in the nose, the application of ether to the nose would stop the respiration immediately, producing passive expiration, or, in other words, *ether would inhibit the inspiratory contractions.*) The respirations were usually reduced in number. The blood pressure was not much below the normal.

The dose of magnesium sulphate which causes complete anæsthesia lies between 1.75 and 1.25 gm. per kilo for the rabbit. A dose above 1.75 per kilo is usually fatal for the animal, and if the place of injection is massaged the fatal issue develops quite rapidly. The animal becomes paralyzed within a few minutes, and respiration ceases before the heart stops; at this stage artificial respiration does not save the animal, as the large dose of magnesium apparently affects also the blood pressure and heart beats, though in a lesser degree. We have lost two animals with only 1.75 gm. per kilo after an energetic massage of the injected area.

A dose of 1.25 per kilo is about the lower limit. Sometimes even a smaller dose will produce an apparently satisfactory anæsthesia and permit an operation, but then stimulation of a sensory nerve would rouse the animal.

The motor impairment seems to precede the anæsthetic influence; when small doses were given, the animal would be unwilling to move around, while the reflexes would still act perfectly.

No anæsthetic dose interfered with the reaction of the pupil to light. A fatal dose would soon cause a dilation of the pupil.

Intramuscular injections.—We did not make numerous intramuscular injections with any of the magnesium salts. We believe, however,

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that we are justified in making the following brief general statement. The effect of an intramuscular injection of magnesium salts is somewhat superior to that of a subcutaneous one, but is by far inferior to the effect of an intravenous application.[1]

Furthermore, the intramuscular injection of these (concentrated) salt solutions seemed to favor incidentally the development of abscesses. Whereas in the subcutaneous injections in rabbits we never had an abscess, sloughing abscesses developed now and then after injections into the muscles. We have to add, however, that the solutions used were not sterilized.

Magnesium Chloride in Rabbits.

In order to prove that the anæsthetic effect which we obtained with subcutaneous injections of magnesium sulphate was due to the magnesium-ion of that salt, we have tested the effect of magnesium chloride after subcutaneous injection. In order to have about the same molecular concentration as the sulphate salt, we used a 19 per cent solution of magnesium chloride — about 2 M. The few experiments we have made, proved beyond doubt that magnesium chloride also exerts a profound anæsthetic effect after subcutaneous injection. With this salt we have not tried to reach the maximum limit; on the contrary, the doses we have used were rarely above 1.0 gm. per kilo, which is about equimolecular with 1.3 gm. of magnesium sulphate, — the latter dose being about the minimum limit for that salt. We will illustrate our experience with one protocol.

Experiment 6. May 9. — Gray rabbit, female, 1100 gm.; respirations about 90 to the minute.

3.40. Injected subcutaneously into right flank 5.5 c.c of a 19 per cent solution of magnesium chloride; massaged.

4.06. Animal unable to move; front leg placed behind ear, stays there.

Animal operated; no resistance and no sign of pain. Cannulæ into trachea, left carotid artery, and left pleural cavity. Left vagus cut. Blood in carotid artery bright red. Operation finished at 4.40. Graphic record of the blood pressure and respirations obtained, and central end of vagus stimulated. (See Fig. 3.)

5.15. Abdomen opened; animal shows no pain, no attempt to press out the intestines from the cavity. No peristalsis of small intestines,

[1] Meltzer and Auer: Journal of experimental medicine, 1905, vii, p. 59.

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which contain liquid and gas. Sciatic nerve stimulated; good contraction of leg, but no pain. Blood still bright red. Animal killed. Bladder contains about 10 c.c. of urine; no reduction with Trommer's test.

This rabbit received only 1 gm. of magnesium chloride per kilo animal. About thirty minutes after injection the animal was operated, and nerves were stimulated without any struggle and without any sign of pain. At the same time respiration was good, blood bright red, and blood pressure nearly normal. No anæsthesia could be deeper and more harmless at the same time.

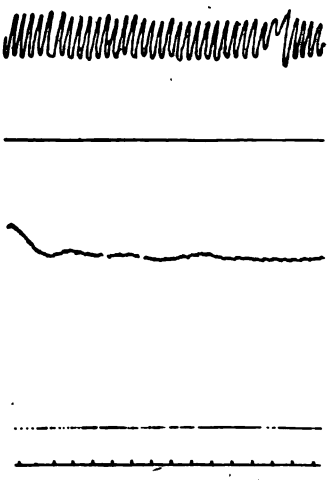

FIGURE 3. — One-half the original size. Tracings obtained from rabbit (Experiment 6) two hours after receiving a subcutaneous injection of 1.0 gm. magnesium chloride per kilo body weight. Tracings and lines the same as in Fig. 1.

SUBCUTANEOUS INJECTIONS OF MAGNESIUM SULPHATE IN CATS.

In a general way our experience with rabbits holds good for cats also. A certain dose of the salt will produce anæsthesia with recovery, while the anæsthesia produced by a larger dose terminates earlier or later in death of the animal. There are, however, some differences between the behavior of the two species towards the subcutaneous injection of magnesium salts. In the first place, the zone of the anæsthetizing non-fatal dose of the salt for the cat is very narrow. For instance, the anæsthesia brought on by a dose of 1.0 gm. per kilo cat terminates fatally, while that produced by 0.8 gm. per kilo was still unsatisfactory. Only a dose of 0.9 gm. of magnesium sulphate produced a satisfactory narcosis from which the animal recovered completely. This statement already contains the further point of difference that cats are very sensitive towards the toxic effect of magnesium salts. Furthermore, the fatal issue, which a larger dose brings on in cats, is mostly preceded by a long stage of deep anæsthesia, at the beginning of which the condition of the animal may be excellent; but the anæsthesia gradually deepens, the respiration becomes more and more slow and shallow; the animal dies without any sign of asphyctic convulsions. The anæsthesia in cats, as a rule,

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sets in much later than in rabbits. The following few protocols will illustrate these statements:

Experiment 7. May 19. — Yellow cat, male, 3820 gm.

3.40 P. M. Injected subcutaneously into left flank 15 c.c. of a 25 per cent solution of magnesium sulphate.

3.45. Respirations 64 to the minute.

4.45. Respirations 128 to the minute, deeper than before (?), salivated, staggers in cage.

4.55. Lying on the floor on one side with relaxed extended extremities; conjunctival reflex present; stepping on tail causes no reaction.

5.25. Lying on the same spot and in the same manner as before; 40 respirations to the minute, moderately deep; conjunctival reflex present; stepping on tail — no reaction; on inserting probe in nose, moves head slightly.

6.00. Respirations 28 to the minute, fairly deep; otherwise no change.

6.22. No change. Observation discontinued.

Next day found dead in the same position as left on the previous evening.

This animal had only 1.0 gm. per kilo of the magnesium salt. In this animal, as well as in animals which received larger doses, the anæsthesia did not set in until about one hour after injection. Then the anæsthesia became profound, lasted long, and terminated finally in death. It is noteworthy that in cats the conjunctival reflex was never fully abolished even when the anæsthesia was so deep that no response was produced by stepping heavily on the tail, cutting the peritoneum, or stimulating sensory nerves.

Experiment 8. May 23. — Male cat, 3330 gm.

3.09 P. M. Injected subcutaneously into left side 10.6 c.c. of a 25 per cent solution of magnesium sulphate, moderately massaged.

4.45. Staggers slightly when walking, which is done in crouching fashion.

5.00. No change; but can be stretched out on board without struggling; not operated. Survived.

Experiment 9. May 26. — Same cat used in last experiment; weighs now 3050 gm. (Lost about 10 per cent of his weight in three days.)

2.45 P. M. Injected subcutaneously in side 11 c.c. of a 25 per cent solution of magnesium sulphate.

3.15. Can still move, but with incoordination and marked weakness. Placed on back and nails clipped without resistance.

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3.45. Tied on board, femoral artery exposed and cannula inserted, crural nerve handled, tail pinched: no sign of pain. Very slight movements of body now and then. Blood pressure about 125 mm. mercury (see Fig. 4).

4.05. Finished taking blood pressure tracing; wound sewed up; no sign of pain. Taken from board and placed on floor; staggered in crouching position to a corner.

May 27. Animal survived. No diarrhœa. Seems well. Did not lose weight within the next three days.

FIGURE 4. — One-half the original size. Blood pressure tracing from cat (Experiment 9), seventy minutes after receiving a subcutaneous injection of 0.9 gm. magnesium sulphate per kilo. Upper curve blood pressure, lower straight line 0-line, interrupted line time in two seconds.

The animal used in the last two experiments was only moderately affected by a dose of 0.8 gm. per kilo (Experiment 8). But with a dose of 0.9 gm. per kilo (Experiment 9) the animal was in a satisfactory state of anæsthesia. It could be operated without any sign of pain. The blood pressure was fairly high, the blood bright red, and the animal survived without suffering any after effects.

It is noteworthy that in cats the anæsthesia set in before the abolition of all voluntary and reflex movements.

Some of the cats vomited at the onset of the anæsthesia. Salivation was not frequent. The urine of the cats which received magnesium sulphate reduced copper sulphate.

Magnesium chloride produced in cats about the same effect as the sulphate. In two young cats doses of 1.0 and 1.2 gm. per kilo produced only slight effects.

No abscesses were produced in cats by subcutaneous injections of the magnesium salts.

Injections of Magnesium Salts in Dogs.

These experiments have demonstrated that this species of animal can also be completely anæsthetized by injections of magnesium salts. In this series we had, however, to contend with some difficulties. In the first place we had to deal with large animals which, in consequence, required large quantities for the production of anæsthesia. In the second place the absorption from the

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subcutaneous tissues of these animals seemed to be distinctly inferior to that of rabbits and cats. Even after considerable massage of the injected place a bulky mass would often remain for hours and even for days and would frequently lead to an extensive abscess. An injection of 1.5 gm. of magnesium sulphate per kilo never led to any satisfactory anæsthesia. A dose of 2 gm. per kilo or a little more would, after some time, anæsthetize the animal, but then the anæsthesia would gradually become very profound and lead finally to death. In fact, of all the experiments in which the dogs received subcutaneously into one place an effective dose of magnesium salts, no animal survived the prolonged, deep anæsthesia into which it gradually passed. The animal would remain for many hours on the same spot and in the same position, and the death was never marked by any struggle. One dog, which received subcutaneously into one place 2 gm. of magnesium sulphate per kilo, and which possibly had a chance of recovery, was utilized for another experimental purpose.[1]

We had more satisfactory results when the injection was distributed in two places,[2] or when the injection was given intramuscularly. For the dog it was quite evident that the absorption from the muscles was better than from the subcutaneous issue.

The following experiments are good illustrations:

Experiment 10. — Male dog, 9300 gm.

2.30 P.M. Injected 65.1 c.c. of a 25 per cent solution of magnesium sulphate, one-half into the muscles of left back and the other half subcutaneously into right side; massaged.

2.37. Vomited.

2.50. Dog flaccid, cannot move, lies on side; stepping on tail — no response. Lid reflex still active.

Stretched on board, head not fixed, femoral artery exposed and blood pressure tracing obtained (see Fig. 5). No sign of pain and no movements whatsoever. Wound sewed up and animal put on floor.

4.15. Dog lies in same position; anæsthesia deeper than ever. No lid reflex.

Next day, dog survived; passed neither urine nor fæces. Five days later had lost nearly one kilo in weight; otherwise apparently normal. Used for another experiment.

[1] That dog, while in profound anæsthesia, received a fatal dose of strychnine for the purpose of studying the condition of his muscles. The animal responded promptly with strong convulsions.

[2] MELTZER: Journal of experimental medicine, 1900–1901, v, p. 643.

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Experiment 11. — Male dog, 8750 gm.

2.55. Injected 61 c.c. of a 25 per cent solution of magnesium sulphate subcutaneously into two separate places (shoulders); massaged well.

3.04. On attempting to move tumbles down in a heap.

3.30. Dog placed on board, femoral artery exposed, cannula inserted; no sign of pain during operation. Blood pressure taken. Frequent slight motions of the animal, each motion causing a short rise of blood pressure (see Fig. 6). Abdomen opened, no sign of pain. Scraping of the parietal peritoneum is resented by movements of the animal; but no definite struggle. Animal killed.

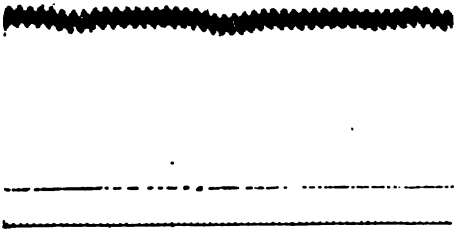

FIGURE 5. One-half the original size. — Blood pressure tracing from dog (Experiment 10) ninety minutes after receiving an injection of 1.75 gm. magnesium sulphate per kilo. One-half of the quantity was given intramuscularly and the other half subcutaneously. Animal perfectly motionless. Curve and lines as in Fig. 4.

Each animal in the two experiments received 1.75 gm. per kilo. In Experiment 10 one-half was given intramuscularly, whereas in Experiment 11 the entire amount was given subcutaneously but in separate places. Both were in satisfactory states of anæsthesia. However, the differences between the degrees of anæsthesia in the two animals are striking. The anæsthesia in the animal of Experiment 10 (partly intramuscular) was already absolutely satisfactory during the operation and the taking of the blood pressure tracings, but later it became still more profound; whereas the anæsthesia of the animal of Experiment 11 was not profound enough during the taking of the tracing and it soon began to show signs of waking up. The difference finds its clear expression in the blood pressure tracings obtained from the two animals. The tracing from the animal of Experiment 11 is frequently interrupted by considerable elevations brought on by the motions of the animal; while that from the animal

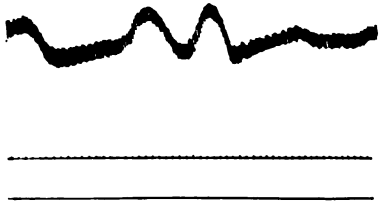

FIGURE 6. One-half the original size. — Blood pressure tracing from dog (Experiment 11) fifty minutes after receiving 1.75 gm. magnesium sulphate per kilo subcutaneously in two different places. Curve and lines as in Fig. 4, time line above base line. Elevations on blood pressure curve correspond to movements of the animal.

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of Experiment 10 runs as evenly as if the animal were also under the influence of curare.

The blood pressure of all dogs which were under anæsthesia from the magnesium salts was comparatively low — under 100 mm. mercury. This apparently did not interfere with the final recovery of the animals. Neither did it seem to be connected with the depth of the anæsthesia, since the blood pressure of dogs with light anæsthesia was also comparatively low.

Nearly all dogs which received magnesium sulphate vomited at least once and in most cases a few times; the first vomiting occurred soon after the injection. This happened even when the general effect was slight.

Dogs which received magnesium chloride did not vomit.

In nearly all cases the injection of magnesium sulphate produced no pain. The injection of magnesium chloride seemed to be slightly painful.

Nearly all the injections in dogs produced abscesses, but the solutions used were not sterilized.

In dogs also, as we have seen above in cats, a satisfactory anæsthesia was fully developed before complete muscular relaxation was attained. Furthermore, a complete abolition of pain was doubtlessly present before the conjunctival reflex was entirely gone.

Injection of Magnesium Sulphate in Guinea-Pigs.

A dose of 1.8 gm. per kilo produced in these animals deep anæsthesia after fifteen minutes; but with this dose death followed about fifteen minutes later. A dose of 0.8 gm. per kilo only quieted the animal. Doses between 1.0 and 1.25 gm. per kilo caused more or less complete anæsthesia followed, as a rule, by complete recovery. However, some individual disposition of the animal seems to have something to do with the degree of the effect. The following protocols will serve as illustrations:

Experiment 12a. — Red guinea-pig, 480 gm.

3.27. Injected 2 c.c. of a 25 per cent solution of magnesium sulphate (about 1.0 gm. per kilo) subcutaneously into right flank.

4.30. Remains on side when placed there; conjunctival reflex slight.

5.00. Able again to get legs under him; can still be held up by a leg with only slight movements of legs and head.

5.30. Resists vigorously being placed on back or side; conjunctival reflex active. Complete recovery.

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Experiment 12b. — Same guinea-pig eight days later, weighs now 535 gm.

2.55. Injected subcutaneously, left flank, 2.4 c.c. of a 25 per cent solution of magnesium sulphate (1.1 gm. per kilo); massaged.

3.20. Can be placed on side to stay; struggles very slightly when held up by one leg; conjunctival reflex slight.

3.44. Continued to lie on back, completely flaccid; corneal reflex practically absent.

3.57. Turned over on belly and moved about. Complete recovery.

Was used two days later for a similar experiment with an identical result.

Experiment 13a. — Black guinea-pig, 595 gm.

2.37. Injected subcutaneously, right flank, 3 c.c. of a 25 per cent solution of magnesium sulphate (1.25 per kilo); slight massage.

4.30. Turns on belly when placed on side; conjunctival reflex active. Five minutes later remains lying on side.

5.00. Still stays on back as placed; slight movements of head and legs when held up by legs.

5.30. Resists being placed on back. Complete recovery.

Experiment 13b. — Same animal (black) eight days later; weighs now 649 gm.

2.58. Injected subcutaneously, right flank, 3 c.c. of a 25 per cent solution of magnesium sulphate (1.2 per kilo); massaged.

3.20. Resists vigorously being placed on side.

3.44. Can be placed on back, but turns on belly again; conjunctival reflex active.

4.45. Resists attempts to be placed on side.

Animal was practically never in a distinct anæsthetic state.

The red guinea-pig of Experiment 12 was completely anæsthetized three times with doses not above 1.1 gm. per kilo; whereas the black animal of Experiment 13 already showed a greater resistance in the first experiment with a dose of 1.25 gm. and was practically not anæsthetized by a dose of 1.2 gm. per kilo.

We have to bear in mind, however, that in guinea-pigs we cannot always be sure that a subcutaneous injection was not partly intramuscular, and hence the variations in the effects with the same doses.

The anæsthetic effect as a rule set in late, often as late as an hour after the injection, and did not last very long. When the effect took place early, the anæsthesia usually had a fatal termination. No intramuscular injections and no magnesium chloride was tried on guinea-pigs.

In no case did the subcutaneous injection of magnesium sulphate produce any diarrhœa.

No abscesses developed in guinea-pigs.

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Injections in White Rats.

Only magnesium sulphate was tested and only by subcutaneous injections.

A dose of 1.25 gm. per kilo had very little effect. 1.50 gm. per kilo had a somewhat better effect and never produced death, but the anæsthesia was far from being complete. Doses of 1.75 gm. per kilo produced complete anæsthesia ten to fifteen minutes after injection; but in five such experiments only two rats survived the deep narcosis; in three animals, the anæsthesia terminated in death half an hour after injection.

Experiment 14. — White rat (red mark on head), 196 gm.

3.20. Injected subcutaneously, left side, 1.2 c.c. of a 25 per cent solution of magnesium sulphate (175 gm. per kilo); massaged.

3.38. Can be placed on back or side to stay; conjunctival reflex absent; no response to pinching tail.

3.50. Respirations labored, slow.

4.15. Turned on his belly, crawling around. Recovered.

Experiment 15. — White rat (blue mark on head), 235 gm.

3.24. Injected subcutaneously, left side, 1.6 c.c. of a 25 per cent solution of magnesium sulphate (1.75 gm. per kilo); massaged.

3.38. Can be placed on back to stay; no response on pinching tail; conjunctival reflex absent.

3.50. Dead; a few moments before heart beats could be distinctly felt, while the respiration had already stopped.

No abscesses developed in the surviving animals.

No diarrhœa followed any injection.

Injections of Magnesium Salts in Frogs.

A dose of 1 c.c. of a 25 per cent solution of magnesium sulphate or of 19 per cent magnesium chloride injected into one of the lymph sacs of a medium-sized frog will cause complete paralysis of the animal within half an hour after the injection, from which it does not recover. The respirations become gradually slower and shallower until they stop entirely. About that time all voluntary and reflex movements completely disappear. The heart beats, which also become slower soon after the injection, stop entirely about two or three hours later. The above dose, with which we started our experiments, is a very large one; we hardly were aware that it amounted to about 8 gm. per kilo frog! Doses of 1.5 to 2.0 gm. per kilo frog can produce

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a complete immobility from which the animal can fully recover sooner or later. The following experiments will serve as illustrations:

Experiment 16. — Frog, 37 gm.

2.30. Injected into ventral lymph sac 0.25 c.c. of a 25 per cent solution of magnesium sulphate (1.5 gm. per kilo).

3.00. Frog lying flat on back as placed; does not move away when pushed; hangs flaccid when lifted by leg. No corneal reflex.

5.00. Turns over actively when placed on back; jumps when pushed.

Next day seems perfectly normal.

Experiment 17. — Frog, 24 gm.; 80 respirations to the minute.

4.00. Injected into dorsal lymph sac 0.19 c.c. of a 25 per cent solution of magnesium sulphate (2.0 gm. per kilo).

4.20. Unable to turn over when on back.

4.35. Flaccid, when raised by leg; no struggle.

5.00. Legs extended, flaccid; 60 respirations to the minute, very shallow.

Next day recovered and normal.

Subcutaneous Injections of Magnesium Sulphate in Fowls.

We made only a few experiments on fowls for the purpose of bringing out the chief point of our contention. Since, however, no experiments were ever made, at least as far as we know, with injections of magnesium salts into fowls, we may be permitted to reproduce here all the protocols of these few experiments.

Experiment 18. — Rooster, 2230 gm.

2.54. Injected subcutaneously, left side, 13.4 c.c. of a 25 per cent solution of magnesium sulphate (1.5 gm. per kilo). Animal responds to each touch with a remonstrating cackle.

3.10. Sits on floor; beak touching floor; quiet; a push causes no cackling.

3.19. Can be placed on back to stay; respiration slow and labored; touch on cornea does not produce the familiar rapid contraction of the third lid.

3.50. In a deep state of anæsthesia; ether applied to nares does not inhibit respiration.

4.20. Clear thin fluid coming from the beak; a large amount of clear fluid issues from the cloaca.

4.48. Death. No preceding struggle.

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Experiment 19a. — Pigeon, 280 gm.

2.50. Injected subcutaneously, right side, 1.7 c.c. of a 25 per cent solution of magnesium sulphate (1.5 gm. per kilo).

(Skin very thin, tore, some of the salt escaped.)

3.06. Pigeon still stands erect; no change.

3.10. Seems a trifle unsteady on legs.

3.19. Crouches now.

3.30. Cannot be placed on back; struggles to regain position.

3.38. Can be placed on back to stay; touch of cornea causes twitch of lid.

3.50. Deep anæsthesia. Ether does not stop respiration.

3.54. Turned over from position on back.

4.48. Attempts to fly.

Next day perfectly well.

Experiment 19b. — Same pigeon two days later; lost weight, 258 gm.; otherwise apparently well.

5.02. Injected subcutaneously, left side upper chest, 1.0 c.c. of a 25 per cent solution of magnesium sulphate (1.00 gm. per kilo); no mishap. Until 6.10 pigeon standing erect, no sign of weakness. Injection had no effect.

Experiment 19c. — Same pigeon four days later; about same weight — 260 gm.

3.05. Injected partly intramuscularly, upper right chest, 1.6 c.c. of a 25 per cent solution of magnesium sulphate (1.5 gm. per kilo).

3.30. Lying on side; no lid reflex; respiration slow and labored; placed on back to stay.

4.00. Still on back, can be raised by one leg, but wings do not hang down.

4.10. Flopped over on legs, but, unable to stand, rests on table, bill touching it.

4.30. Lying on side, bill touching table. Tossed into air, made ineffectual attempts to fly. Seems to wake up more; can no longer be placed on back, slight lid reflex.

5.35. Sitting, but legs do not seem to be able to bear weight of body.

Next day normal.

Experiment 20a. — Chicken, 1590 gm.

4.58. Injected subcutaneously, left chest, 6.4 c.c. of a 25 per cent solution of magnesium sulphate (1.0 gm. per kilo).

5.35. Can no longer stand; sits; can be placed on side.

5.50. No change; large amount of fluid passed from cloaca.

6.10. Again a good deal of fluid passed from cloaca. Otherwise no change.

Next day normal. Had henceforth loose stools.

Experiment 20b. — Same chicken; lost weight, now 1548 gm.

3.02. Injected subcutaneously, right chest, 9.3 c.c. of a 25 per cent solution of magnesium sulphate (1.5 gm. per kilo).

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3.20. Drowsy; prefers to squat with eyes closed.

3.33. Squatting on table; passed a good deal of clear fluid from cloaca.

4.00. Again passed a good amount of fluid. Can now be placed on side to stay; beak on floor.

4.30. No change; lid reflex active.

5.25. Can still be placed on back, but turns over after short time.

Next day apparently well.

These experiments on fowls have demonstrated that about 1.0 gm. of magnesium sulphate per kilo had either no effect, as in the pigeon, or very little effect, as in the chicken; and further that 1.5 gm. per kilo might produce a moderate anæsthetic effect, as in the chicken, or a profound anæsthesia with perfect recovery, as in the pigeon (partly intramuscular), or a profound anæsthesia with a fatal termination, as in the rooster.

In other words, magnesium sulphate can produce a profound anæsthesia, leading either to perfect recovery or to death, without any symptoms of excitation.

Summary.

We shall now briefly recapitulate our results. In the different species of animals with which we have experimented, rabbits, cats, dogs, guinea-pigs, white rats, frogs, and fowls, we found without any exception:

1. *That a certain dose of magnesium sulphate will produce a deep, often long lasting anæsthesia with complete relaxation of all the voluntary muscles and abolition of some of the less important reflex activities, which anæsthesia terminates in perfect recovery.*

2. *That a large dose of magnesium salt will produce a profound anæsthesia and general paralysis which leads sooner or later to a calm death without being preceded or accompanied by any symptoms of excitation.* Furthermore, in all of the many experiments we have made with magnesium sulphate, *not a single instance was observed in which that salt produced an increase of excitation;* on the contrary, *any effect which this salt produced was invariably in the direction of a reduction of excitation or of its complete temporary or permanent abolition.*

The same was true also of magnesium chloride in the instances in which it was tested.

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With the exception of cats, for all animals a dose of 1.0 gm. of magnesium sulphate per kilo or less had very little effect; a dose of a little over 2 gm. per kilo brought on a fatal anæsthesia. The doses lying between 1 and 2 gm. per kilo animal were the ones which produced more or less complete anæsthesia with recovery. In one species of animal the anæsthetic dose lay nearer the 1 gm. per kilo, and in another species it was nearer the 2 gm. per kilo limit. In general it can be stated that for nearly all animals the dose of magnesium sulphate which can produce anæsthesia with recovery by subcutaneous injection is 1.5 gm. per kilo animal.

For cats it was found that 0.9 per kilo was about the anæsthetic dose for an adult animal. Doses of magnesium sulphate below 0.9 per kilo produced but little effect, and the anæsthesia following a larger dose had a fatal termination. Younger animals seemed to stand a larger dose.

Intramuscular injections, or the injections of divided doses into several places, have favored in some instances the onset of the anæsthetic effect.

We have to mention here that the impression we gained from all experiments was that the animals which urinated frequently had a better chance for recovery. Early urination seemed also to prevent the full development of anæsthesia. Furthermore it seemed that magnesium sulphate after subcutaneous injection acted as a diuretic. Urination probably carried off some of the salt and prevented its fatal accumulation in the blood.

In cats and especially in dogs the injection of magnesium sulphate produced in many instances vomiting or retching. This occurred long before the onset of anæsthesia, and in some instances even when no anæsthesia followed. In the experiments with magnesium chloride no vomiting was observed.

Except in the one chicken we have never seen that the subcutaneous injection of magnesium sulphate ever led to an immediate or late appearance of diarrhœa, or even simply to more frequent stools. In two or three instances in which the abdomen was opened during the experiment or immediately upon its termination there was some fluid and gas present in the gut, but especially in the small intestines. This observation deserves attention with regard to the older and recently renewed claims that subcutaneous injections of magnesium sulphate act as a purgative.

Subcutaneous injection of a 25 per cent solution of magnesium

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sulphate seemed to produce hardly any pain in the animals.[1] Magnesium chloride solutions (19 per cent) seemed to be more irritating.

The subcutaneous injection of large quantities of the salt solutions produced abscesses in dogs which healed promptly. In these experiments, as already stated above, no aseptic precautions were taken. In the many injections given to rabbits (with one or two exceptions after intramuscular injection), guinea-pigs, white rats, fowls, and even cats, abscesses were very rarely observed. It remains to be seen in future studies whether by sterilization and asepsis such abscesses can be avoided.

We may in this connection perhaps be permitted to add that the exclusive object of this study was to establish the essential principle, namely, that magnesium salts possess strong anæsthetic powers. The practical application of this new principle was not taken into consideration during the present study.

The fatal effect of the magnesium salts was produced in the first place by paralysis of respiration. It became slower and somewhat labored and with active expirations even in the non-fatal states of anæsthesia. In the fatal anæsthesias the respiration gradually became very slow and very shallow, until finally it stopped entirely. The heart usually continued beating for a while after the cessation of respiration. The institution of artificial respiration at this stage, however, never resuscitated the animal.

In rabbits and cats the blood pressure during the anæsthesia was very little depressed. In dogs it appeared to be considerably lowered. However, it did not prevent prompt recovery.

It deserves to be mentioned especially that in rabbits, even during deepest anæsthesia by magnesium salts, the application of ether to the nose inhibited the inspiration immediately. In anæsthesia by ether or chloroform this occurs only at the beginning, but not when the animal is already fairly under the influence of the anæsthetic. We shall for the present not enter into a full discussion of the interpretation we are inclined to give to this phenomenon. We shall only remind that the stoppage of respiration by ether, etc., is generally considered as an act of reflex inhibition of inspiration by means of stimulation of the peripheral ends of the second branch of the trigeminus. We shall then state our observation in the following

[1] One of us received twice, subcutaneous injections of small doses (1.0 and 1.5 c.c.) of the 25 per cent solution of magnesium sulphate. It produced practically no pain or irritation worth mentioning.

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words: Magnesium salt, which reduces or temporarily abolishes (inhibits) many reflex acts, does not interfere with the trigeminal reflex-inhibition of respiration.

Finally, in cats and also in dogs a state of deep anæsthesia, absence of pain sensation, and complete muscular relaxation can be fully developed before the stage of complete abolition of the conjunctival reflex is reached. The latter stage, especially in cats, seems to border on the danger line. Furthermore, in these animals the stage of complete anæsthesia (absence of the sensation of pain) seems to be reached before there is a complete abolition of all voluntary movements. These animals could be operated upon without any sign of pain or resistance while they were still able to move about somewhat.

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